Fattori antropici e biologici che influenzano i processi di decomposizione del legno morto in specie forestali

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To Gino

A dog will teach you unconditional love.

(Robert Wagner)

Indice

Riassunto

Per legno morto (Coarse Woody Debris; CWD) si intende il residuo di alberi vivi, inclusi alberi caduti a terra, rami caduti e frammenti legnosi presenti sul suolo forestale. Il CWD rappresenta una componente importante per il funzionamento degli ecosistemi forestali, influenzando il ciclo dei nutrienti, la formazione dell'humus e lo stoccaggio del carbonio. È considerato un elemento chiave per il mantenimento della biodiversità forestale, rappresentando un microhabitat per centinaia di specie di invertebrati, funghi, briofite, licheni, anfibi, piccoli mammiferi e uccelli. Negli ecosistemi forestali la quantità di CWD può variare in relazione alla tipologia forestale, alla gestione del popolamento ed ai disturbi naturali. In particolare, nelle foreste semi-naturali, la quantità di CWD è il risultato della mortalità arborea, mentre nelle foreste gestite, tale quantità è anche influenzata dalle attività di taglio e dalle pratiche di gestione (ad es. ceppaie e ramaglie rilasciate al suolo). Tuttavia, come conseguenza dei cambiamenti climatici in atto, che possono indurre un aumento dei tassi di decomposizione, è prevista una sua riduzione nel lungo periodo. Comprendere le fluttuazioni della quantità del CWD e dei relativi tassi di decomposizione è un aspetto molto importante per analizzare più in dettaglio le dinamiche legate al ciclo dei nutrienti e del carbonio negli ecosistemi forestali. Sulla base di tali considerazioni, obiettivo principale dell'elaborato di tesi è stato quello di indagare diversi fattori antropici (es gestione forestale) e biologici (es funghi) che influenzano l'abbondanza ed i processi di decomposizione del legno morto in due specie forestali quali il faggio (*Fagus sylvatica* L.) e l'abete bianco (*Abies alba* Mill.). Il lavoro è strutturato in tre capitoli, in cui nel primo vengono analizzati a scala di popolamento gli effetti a breve termine di pratiche selvicolturali innovative e tradizionali sulla presenza e distribuzione del legno morto e su altri indicatori di biodiversità (es. microhabitats e vegetazione del sottobosco) in tre faggete (*Fagus sylvatica* L.) montane dislocate lungo un transetto latitudinale in Italia. Nel secondo e nel terzo capitolo vengono invece analizzati i processi di decomposizione del legno morto, spostando quindi l'approccio sperimentale ad una scala di maggior dettaglio. In particolare, nel secondo capitolo viene analizzato il profilo degli zuccheri semplici nel legno vivo di abete bianco e nei campioni di CWD raccolti nelle cinque fasi di

decadimento di Hunter in due periodi stagionali diversi, all'interno di due siti forestali alpini italiani (TN). Nel terzo capitolo viene invece effettuata un'analisi dei composti fenolici quali prodotti di degradazione della lignina nel legno di abete bianco prima e dopo l'azione enzimatica di nove funghi del marciume bianco, di cui sei appartenenti al genere *Armillaria spp*. (*A. borealis, A. cepistipes, A. gallica, A.mellea, A. ostoyae*, e *A. tabescens*) e tre al genere *Heterobasidion spp*. (*H. abietinum, H. parviporum* e *H. annosum sensu stricto*) in condizioni controllate in laboratorio. I risultati del primo lavoro hanno evidenziato diversi effetti dei trattamenti selvicolturali sugli indicatori analizzati (es. legno morto, microhabitats e vegetazione del sottobosco). In particolare, l'applicazione dei trattamenti innovativi (CTT), rispetto ai tradizionali (LT) ha comportato in tutte e tre le aree di studio indagate, un aumento significativo del volume di legno morto e degli altri indicatori esaminati, sottolineando l'importanza di tali trattamenti come valida alternativa gestionale per le faggete montane del Mediterraneo, in termini di conservazione della biodiversità. La seconda attività ha rappresentato un primo tentativo di analisi della produzione di carboidrati durante il processo di decomposizione del legno morto di abete bianco. Dai risultati ottenuti è stato possibile osservare differenze negli andamenti e nella variabilità del contenuto degli zuccheri semplici all'avanzare del processo di decomposizione del legno morto in campo, probabilmente legate alle diverse condizioni ambientali (es. temperatura e umidità) che caratterizzano i due siti indagati e all'azione degli organismi decompositori come i funghi. I risultati del terzo lavoro hanno infine evidenziato diverse strategie di accumulo e degradazione dei composti fenolici della lignina nel legno di abete bianco da parte di nove specie fungine dei generi *Armillaria spp.* e *Heterobasidion spp*., mediante la secrezione degli enzimi ligninolitici, sottolineando l'importanza di tali organismi nel processo di decomposizione.

In definitiva, il presente elaborato può essere considerato un contributo utile ad incrementare le conoscenze scientifiche inerenti la complessità delle relazioni che intercorrono tra il legno morto e i diversi fattori che ne influenzano l'abbondanza ed i processi di decomposizione, con particolare riferimento a due specie forestali europee di ampia diffusione, quali il faggio e l'abete bianco.

Abstract

Coarse woody debris (CWD) is the residue of living trees including fallen trees, fallen branches and lying woody debris (CWD). CWD is an important component of functioning of forest ecosystems, influencing the nutrient cycling, the humus formation and the overall forest carbon storage. It is a key element for forest biodiversity, representing a microhabitat for hundreds of species of invertebrates, fungi, bryophytes, lichens, amphibians, small mammals and birds. In forest ecosystems, the amount of CWD can largely varies in relation to forest types, stand management and natural disturbances. In particular, in semi-natural forests, the amount of CWD is the result of tree mortality while, in managed forests, logging and management practices also influence CWD (e.g. left-over stumps and branches). However, as a consequence of the climate warming, a decrease in CWD could be expected, due to the enhanced decomposition rates. Understanding the fluctuations in CWD abundance and the related decay rates is very important to better understand the nutrient cycling and carbon dynamics of forest ecosystems. Based on these considerations, the primary objectives of this thesis were to evaluate the effects of anthropic (e.g. forest management) and biological factors (e.g. fungi) on deadwood abundance, distribution and decomposition in two European tree species such as beech (*Fagus sylvatica* L.) and silver fir (*Abies alba* Mill.). The present work is structured in three chapters. In particular, in the first chapter, the short-term effects of innovative and traditional silvicultural treatments on the abundance and distribution of deadwood and other indicators of biodiversity (e.g. microhabitats and undergrowth vegetation) were analysed at a stand-scale in three mountain beech forests located along a latitudinal transect in Italy. In the second and third chapters, the decomposition processes of silver fir deadwood were analysed, moving the experimental approach to a more detailed scale. In particular, in the second chapter, the sugar profiles of living silver fir trees and CWD samples belonging to Hunter's five decay stages was studied in two different seasonal periods and in two Alpine forests located in Northern Italy (TN). In the third chapter, a phenolic compounds analysis was carried out in silver fir wood before and after the enzymatic action of nine white rot fungi, belonging to *Armillaria spp*. (*A. borealis, A. cepistipes, A. gallica, A. mellea, A. ostoyae,* and *A.*

tabescens) and *Heterobasidion spp*. (*H. abietinum*, *H. parviporum* and *H. annosum sensu stricto*) genera in controlled lab conditions. Results from the first research showed different effects of silvicultural treatments on the analysed indicators (e.g. deadwood, microhabitats and understory vegetation). In particular, the application of the innovative treatments (CTT), compared to the traditional ones, involved a significant increase of deadwood volume and other biodiversity indicators (e.g. microhabitats and understory vegetation), in all the three study areas, highlighting the importance of these treatments as a valid management option for Mediterranean beech forests in terms of biodiversity conservation. The second study was a preliminary attempt to investigate the production of carbohydrates during deadwood decay in field conditions. The results showed differences in trends and variability of simple sugar contents during deadwood decomposition in the field, probably related to the different environmental conditions (e.g. temperature and humidity) that characterize the two Alpine sites and by the action of decomposing organisms such as fungi. Finally, the results of the third research highlighted different strategies of phenolic compounds accumulation and consumption in lignin of silver fir wood after the action of nine fungal species belonging to *Armillaria spp*. and *Heterobasidion spp*. genera, through the secretion of ligninolytic enzymes, emphasizing the importance of these organisms during decomposition process. Ultimately, this thesis represents a useful contribution to increase the scientific knowledge on the complex relationships between deadwood and environmental factors influencing its abundance and decay processes, focusing on two widespread European tree species, such as beech and silver fir.

INTRODUZIONE GENERALE

Introduzione

In Europa, le foreste occupano circa 182 milioni di ettari (il 43% della superficie terrestre dell'UE), ospitando il maggior numero di specie animali e vegetali del continente (Toscani and Sekot, 2018). Esse svolgono importanti funzioni ecologiche, economiche e sociali tra cui: la conservazione ed il mantenimento della biodiversità forestale, la fissazione dell'anidride carbonica con la conseguente mitigazione dei fenomeni di riscaldamento globale, la protezione delle risorse idriche e pedologiche, la produzione di prodotti forestali legnosi e non, ma anche funzioni turistico-ricreative a beneficio dell'intera collettività (Mori and Gustafsson, 2017; Palacín et al., 2017; Pohjanmies et al., 2017). L'importanza delle foreste e del ruolo multifunzionale che esse ricoprono rappresentano tematiche rilevanti trattate già a partire dagli anni '70 (Conferenza di Stoccolma nel 1972) in diverse iniziative nazionali ed internazionali (es. Vertice della Terra di RIO92 nel 1992, Conferenze ministeriali per la Protezione delle Foreste in Europa), nelle quali l'obiettivo principale è stato ed è ancora oggi la continua ricerca di indicatori e linee guida volti a garantire una corretta ed attenta gestione dei sistemi forestali (Ness et al., 2007; Diaz-Balteiro et al., 2017; Kraxner et al., 2017; Kates, 2018). In particolare, nel corso della seconda Conferenza Ministeriale per la Protezione delle Foreste in Europa (Helsinki, 1993), al fine di valorizzare le diverse funzioni svolte dalle foreste, è stato definito per la prima volta il concetto di Gestione Forestale Sostenibile (GFS), inteso come "la gestione e l'uso delle foreste e dei terreni forestali nelle forme e ad un tasso di utilizzo che consentano di mantenerne la biodiversità, produttività, capacità di rinnovazione, vitalità e potenzialità e di adempiere, ora e nel futuro, a rilevanti funzioni ecologiche, economiche e sociali a livello locale, nazionale e globale, senza comportare danni ad altri ecosistemi". Emerge da questa definizione come il cuore della GFS sia la conservazione della funzionalità degli ecosistemi forestali in un'ottica di interazione dinamica con gli altri ecosistemi naturali. In molti paesi sviluppati, la crescente attenzione ai valori ambientali ed al ruolo multifunzionale delle foreste in termini non solo produttivi, ma anche conservativi, ha portato ad una riduzione dell'intensità di sfruttamento delle stesse e a modifiche delle pratiche selvicolturali tradizionali con metodi di gestione più attenti a preservare i sistemi biologi forestali.

Inoltre, secondo le rivelazioni di un rapporto della FAO (*The Global Forest Resources Assessment 2015*), dal 1990 ad oggi, grazie anche a legislazioni più efficienti ed un maggior coinvolgimento delle comunità locali nello sviluppo delle politiche destinate alle foreste, il tasso di deforestazione netto si è ridotto di oltre il 50% rispetto al passato. Al fine di poter valutare e quantificare gli effetti di una gestione forestale sostenibile, nel corso della terza Conferenza Ministeriale per la Protezione delle Foreste in Europa (MCPFE, 2003) sono stati definiti e messi a punto sei criteri ed una serie di indicatori quali-quantitativi Pan Europei di Gestione Forestale Sostenibile (Paletto et al., 2014). Tra i vari indicatori, il legno morto è stato espressamente citato e politicamente riconosciuto (Nine improved Indicators under the Criterion 4: «Maintenance, Conservation and Appropriate Enhancement of Biological Diversity in Forest Ecosystems», MCPFE, 2003) come un importante strumento ed un valido indicatore di monitoraggio della biodiversità e della naturalità degli ecosistemi forestali (Verkerk et al 2011; Forest Europe, 2015; Kunttu et al., 2015). Nel corso degli anni, l'attenzione sull'importanza del legno morto e le sue relazioni con le cenosi forestali è stata oggetto di studio e continui approfondimenti ed il suo rilevamento è stato inserito nei principali programmi di monitoraggio (Travaglini et al., 2007a), inclusi gli inventari forestali nazionali.

Il legno morto ed il suo ruolo ecologico all'interno degli ecosistemi forestali

A livello terminologico il *Global Forest Resources Assessment* del (2005) definisce il legno morto come tutta la biomassa legnosa non vivente, non contenuta nella lettiera, sia essa in piedi, a terra o nel suolo, includendo in tale definizione gli alberi interi o frammenti di legno a terra, alberi morti in piedi, gli "snag", le radici morte e le ceppaie purché superiori ad una soglia dimensionale prestabilita pari a 10 cm (FAO, 2004). In relazione alle dimensioni, la necromassa può essere inoltre suddivisa in detriti legnosi grossolani (CWD) con un diametro medio maggiore di 10 cm, in detriti legnosi fini (FWD), con diametro medio compreso tra 5 e 10 cm, e molto fini (VFWD), con diametro medio inferiore a 5 cm (Densmore et al., 2005, Küffer and Senn-Irlet, 2005b, Juutilainen et al., 2014). La presenza del legno morto, nelle opportune proporzioni commisurate anche alle finalità di coltivazione della foresta, risulta di fondamentale importanza per il funzionamento degli ecosistemi forestali (Gao et al., 2015; Parisi et al., 2018). Esso rappresenta un elemento chiave per la conservazione ed il mantenimento della biodiversità forestale (Stokland et al., 2012; Moghimian et al., 2017; Tavankar et al., 2017), rappresentando il microhabitat per centinaia di specie di invertebrati (Parisi et al., 2018) e vertebrati, compresi anfibi (Blomquist and Hunter, 2010), piccoli mammiferi (Fauteux et al., 2012) ed uccelli (McComb and Lindenmayer, 1999; Redolfi De Zan et al., 2014) che utilizzano la necromassa legnosa come fonte di nutrimento, riparo e nidificazione (Mac Nally et al., 2001; Fauteux et al., 2012). Il legno morto rappresenta inoltre un habitat ottimale ed un substrato di crescita e nutrimento per numerosi organismi, compresi insetti e funghi decompositori (Boddy et al., 2008; Lassauce et al., 2011; Bouget et al., 2012; Stokland et al., 2012; Parisi et al., 2018; Purahong et al., 2018), briofite, licheni epifitici (Andersson and Hytteborn, 1991; Spribille et al., 2008) e specie erbacee (Szewczyk and Szwagrzyk, 1996). Oltre alla conservazione della biodiversità, il legno morto svolge un ruolo chiave nella mitigazione dei cambiamenti climatici mediante lo stoccaggio ed il lento rilascio del carbonio organico (Laiho and Prescott 1999; Strukelj et al., 2013; Petrillo et al., 2015; Hadden et al., 2017), rappresentando insieme alla lettiera, al suolo ed alla biomassa epigea ed ipogea, uno dei cinque pool di carbonio degli ecosistemi forestali (IPCC, 2003). Esso inoltre svolge un ruolo

importante nella conservazione e nel miglioramento della fertilità del suolo (Stockli, 1996), favorendo la formazione di humus (Berg and McClaugherty, 2003; Fravolini et al., 2016) ed il rilascio di sostanze nutritive (Prescott et al., 1993; Holub et al., 2001) utili alla rinnovazione naturale (Stockli, 1996) oltre che rappresentare siti idonei all'insediamento di quest'ultima (Orman et al., 2016). Nel breve e medio periodo, può infine favorire la stabilità dei versanti preservandoli dal rischio idrogeologico e dai fenomeni di erosione (Hagan and Grove 1999; Bobiec, 2002).

I fattori che influenzano l'abbondanza e la distribuzione del legno morto in foresta

Negli ecosistemi forestali, la quantità di legno morto è molto variabile e può essere influenzata da diversi fattori tra cui: il tipo e lo stadio di sviluppo dei popolamenti forestali, l'intensità e la frequenza dei disturbi naturali (es. eventi metereologici, incendi naturali, attacco da parte di funghi ed insetti), le condizioni climatiche locali, i tassi di decomposizione (Behjou et al., 2018) e le modalità ed intensità delle pratiche di gestione selvicolturali (Lombardi et al., 2008; Larrieu et al., 2012; Perry et al., 2013). In particolare, nelle foreste naturali delle diverse regioni d'Europa, i quantitativi di legno morto sono determinati principalmente dalla mortalità degli alberi in seguito a processi di senescenza e competizione (Merganičová et al., 2012; Lombardi et al., 2010). Inoltre, in tali ecosistemi, la quantità del legno morto può essere influenzata anche dalla frequenza e dall' intensità dei disturbi naturali abiotici (es. vento, fuoco ecc.) e biotici (es. insorgenza di insetti, attacchi fungini ecc.; Hahn and Christensen, 2004; Rahman et al., 2008) e dalla scala spaziale alla quale essi si manifestano. Di solito, i disturbi naturali su piccola scala si verificano con maggiore frequenza causando la morte di singoli o piccoli gruppi di alberi, comportando quindi un rilascio continuo di legno morto negli ecosistemi forestali (Rahman et al., 2008). I disturbi naturali a scala più ampia influenzano l'intero ecosistema (Merganičová et al., 2012) portando ad un rilascio di legno morto variabile in relazione alla fase del disturbo (Rahman et al., 2008; Bače et al., 2015). Differenti accumuli di legno morto si verificano anche in relazione al tipo ed allo stadio di sviluppo dei popolamenti forestali. Nei boschi di latifoglie, rispetto ai boschi di conifere, si riscontrano in genere minori quantitativi di legno morto, anche a causa della maggiore velocità di decomposizione del materiale legnoso (Peterken, 1996; Christensen et al., 2005). Riguardo lo stadio di sviluppo dei popolamenti, alcuni studi hanno dimostrato la presenza di quantitativi di legno morto inferiori nei popolamenti forestali maturi, rispetto a quelli negli stadi giovanili (Merganičová et al., 2004; Merganičová and Merganič, 2010). Oltre ai fattori naturali discussi finora, negli ecosistemi forestali, la quantità di legno morto può essere influenzata anche dai disturbi antropici, come l'intensità e le modalità di realizzazione delle pratiche selvicolturali (Guby and Dobbertin, 1996, Green and Peterken, 1997; Vítková et al., 2018). Numerosi

studi relativi alla stima dei quantitativi di legno morto in relazione alle pratiche di gestione forestale sono stati effettuati in Europa ed in Nord America (Guby and Dobbertin 1996; Siitonen et al., 2000; Pedlar et al., 2002; Lombardi et al., 2008). I risultati hanno evidenziato quantitativi di legno morto inferiori nelle foreste gestite (Lombardi et al., 2008; Stokland et al., 2012; Dieler et al., 2017; Nagel et al., 2017) rispetto a quanto rilevato nelle foreste naturali o gestite secondo i principi di GFS (Kirby et al., 1998). In generale, solo il 2-30% dell'ammontare di legno morto presente nelle foreste naturali si ritrova in quelle soggette ad interventi selvicolturali (Green and Peterken 1997, Kirby et al., 1998, Jonsson, 2000). Nelle foreste semi-naturali dell'Europa, Nilsson et al., (2002) hanno rilevato quantitativi di legno morto pari a 130-150 m^3 ha^{-1}, rispetto a quelli presenti in seguito allo sfruttamento antropico, con valori compresi tra 1 e 23 m^3 ha^{-1} (MCPFE, 2007). Altri studi hanno dimostrato che, mentre nelle foreste naturali di faggio (Commarmot et al., 2013) ed abete rosso (Ranius et al., 2003), la quantità di legno morto era pari al 30% o addirittura al 40% del volume totale, nelle foreste europee gestite, tale percentuale era in genere inferiore al 5% (Bütler and Schlaepfer 2004; MCPFE, 2007). In altri studi, condotti in diverse faggete montane in Europa, i volumi di legno morto rilevati sono risultati molto variabili, ma comunque superiori rispetto a quelli presenti nelle faggete gestite (Green and Peterken, 1997; Paletto et al., 2014). Ad esempio, in uno studio condotto nelle faggete montane italiane, Lombardi et al., (2012) hanno riportato un volume medio di legno morto pari a 60 m^3 ha^{-1}, compreso tra 2 e 143 m^3 ha^{-1}. Vandekerkhove et al., (2009) in uno studio condotto nelle foreste di pianura dell'Europa nord-occidentale e centrale, hanno registrato un volume medio di legno morto pari a 53 m^3 ha^{-1}, compreso tra 6 e 500 m^3 ha^{-1}. Inoltre, grandi quantità di legno morto (> 100 m^3 ha^{-1}) sono state rilevate in riserve forestali di faggio francesi (Mountford, 2002), nelle foreste submontane ungheresi (Odor and Standovar, 2003), nel parco nazionale Krkonoše della Repubblica ceca (Vacek et al., 2015) e nelle riserve forestali di faggio dei Carpazi nord-occidentali (Kucbel et al., 2012). I volumi di legno morto rilevati nelle foreste di faggio gestite sono invece risultati inferiori, pari a 5 m^3 ha^{-1} nella Spagna orientale (Hernando et al., 2013); inferiori a 10 m^3 ha^{-1} in Boemia centrale (Bilek et al., 2011) e compresi tra 8,8 e 47,1 m^3 ha^{-1} in diversi distretti forestali

in Italia (Paletto et al., 2012). Inoltre, in un recente studio relativo alla distribuzione del volume medio di legno morto in relazione alla gestione dei popolamenti forestali, realizzato in 19 paesi in Europa, Puletti et al., (2017) hanno evidenziato alti quantitativi di necromassa (> 100 m^3 ha^{-1}) nelle regioni montuose dell'Europa centrale gestite ad alto fusto, e quantitativi inferiori in aree forestali della regione mediterranea e parte della Gran Bretagna gestite a ceduo. Come dimostrato dai diversi studi, i minori quantitativi di legno morto rilevati nelle foreste gestite rispetto a quelle non gestite (Green and Peterken, 1997; Paletto et al., 2014) potrebbero derivare dall'applicazione di pratiche selvicolturali intensive con finalità esclusivamente produttive (Nillson et al., 2001; Keren and Diaci, 2018) e alla rimozione di ramaglie e altri residui di lavorazione per ridurre al minimo la presenza di ostacoli fisici alle attività selvicolturali (Priewasser et al., 2013, Michalová et al., 2017). La stima delle diverse componenti del legno morto in un determinato habitat, in relazione alla tipologia forestale ed alla gestione, risulta essenziale per la scelta di opzioni di gestione forestale sostenibili (Lombardi et al., 2008). Pertanto, nell'ottica di una selvicoltura sostenibile rivolta alla salvaguardia dei processi naturali e alla conservazione della complessità ecosistemica, si stanno attuando politiche gestionali finalizzate ad incrementare i quantitativi di legno morto nelle aree forestali gestite (Hodge and Peterken, 1998, Harmon, 2001). Esiste un gran numero di pubblicazioni in merito alle quantità di legno morto che dovrebbero essere rilasciate nelle foreste in seguito alle utilizzazioni forestali. Mentre alcuni studi suggeriscono il rilascio di almeno 3 m^3 ha^{-1} (Utschik, 1991) o 5-10 m^3 ha^{-1} (Ammer, 1991), altri studi più recenti suggeriscono un rilascio maggiore, con valori compresi tra i 15 ed i 30 m^3 ha^{-1} (Bütler and Schlaepfer, 2004; Colak, 2002; Müller and Bütler, 2010). Oltre alle pratiche di gestione selvicolturali, la quantità e distribuzione del legno morto in foresta può essere influenzata anche dalle condizioni climatiche. In particolare, il clima rappresenta un fattore determinante nel processo di decomposizione del legno morto (Russel et al., 2014). Alcuni studi hanno dimostrato che l'aumento delle temperature medie nel lungo periodo, come conseguenza del riscaldamento climatico in atto, ha portato ad un aumento dei tassi di decomposizione (Mazziotta et al., 2014; Garbarino et al., 2015) e ad una diminuzione significativa del volume di legno morto

all’interno degli ecosistemi forestali (Bradford et al., 2014; Russel et al., 2014); di conseguenza, è stata alterata anche l’abbondanza, la composizione e l’attività delle comunità di organismi decompositori (Singh et al., 2010; Asemaninejad, 2016).

I processi di decomposizione del legno morto

I meccanismi di decomposizione del legno morto sono guidati da processi fisici, chimici e biologici (Harmon et al.,1986) complessi dal punto di vista ecologico, che nel tempo possono influenzare la presenza e l'abbondanza del legno morto all'interno degli ecosistemi forestali (Merganičová et al., 2012; Lombardi et al., 2013). Tali meccanismi sono principalmente caratterizzati da tre fasi sinergiche tra loro: la respirazione eterotrofa, la lisciviazione e la frammentazione (Zhou et al., 2007), durante le quali si verifica una diminuzione delle dimensioni, una perdita di massa e cambiamenti nella composizione chimica del materiale legnoso. In particolare, la fase di respirazione eterotrofa avviene in seguito all'invasione della necromassa da parte di diverse specie di invertebrati saprofiti, tra cui batteri, insetti e funghi, il cui attacco comporta una perdita della massa iniziale ed un rilascio di circa il 50% di carbonio organico come CO_2, ma anche di H_2O e nutrienti (Spies et al., 1988). Le perdite di carbonio sotto forma di CO_2 ed il rilascio di materia organica in seguito a processi di respirazione possono variare in relazione alla quantità ed all'attività metabolica degli organismi decompositori (Li, 1992a). Le varie attività svolte dagli organismi decompositori possono inoltre subire variazioni in relazione al periodo stagionale e allo stadio di decomposizione del detrito legnoso, risultando maggiori e più intense nel periodo estivo (Li, 1992a) e nella necromassa afferente a stadi di decomposizione più elevati (Ausum, 1977; Zhou et al., 2007). All'avanzare dei processi di decomposizione del legno morto, oltre alla fase di respirazione eterotrofa, la lisciviazione concorre ai processi decompositivi (Swift et al., 1979). Essa viene intesa come un processo chimico-fisico operato dalle acque meteoriche (acqua che percola attraverso il tronco), che implica la rimozione di nutrienti e materiali solubili dalla ulteriore frammentazione della struttura del legno e riduzione della biomassa (McMinn and Crossley, 1993). Generalmente, tale processo assume particolare rilevanza nelle fasi più avanzate di decomposizione in cui, oltre all'azione degli agenti atmosferici, l'attività metabolica dei microrganismi decompositori comporta una maggiore liberazione di composti solubili provenienti dalla degradazione dei polimeri del legno, tra cui cellulosa, emicellulosa e lignina (Harmon et al., 1986 Spears and Lajtha, 2004; Fravolini et al., 2018). I composti solubili liberati

possono essere assimilati dagli apparati radicali, immobilizzati nel terreno, o divenire substrati utili per il metabolismo dei micro-organismi presenti nel suolo. La fase di frammentazione completa ed intensifica il processo di degradazione del legno. In particolare, tale fase si compone di una serie di processi chimici, fisici e biologici che portano, nel corso della degradazione, ad una riduzione significativa di massa e volume del detrito legnoso. La frammentazione può essere di tipo fisico o biologico (Harmon et al., 1986). La prima, causata da fattori fisici quali agenti atmosferici, tra cui vento, neve e pioggia, consiste nella rottura e disgregazione del materiale legnoso durante o in seguito alla sua caduta al suolo. Tale tipo di frammentazione comporta modificazioni della superficie esterna del detrito legnoso, visibili dall'insorgenza di fessure. La frammentazione di tipo biologico interessa invece la parte interna del legno: essa è causata principalmente dall'azione degli organismi decompositori, tra cui numerose specie di insetti e funghi (Harmon et al., 1986). Le varie fasi responsabili della decomposizione del legno morto possono verificarsi con diversa intensità, comportando variazioni dei tassi di degradazione, in relazione all'influenza di diversi fattori abiotici e biotici (Bani et al., 2018), tra cui le caratteristiche del substrato legnoso, il rapporto corteccia - legno, le dimensioni del substrato, la specie arborea, il contatto con il suolo minerale (Van der Wal et al., 2007), le condizioni micro-climatiche locali (temperatura e umidità; Mackensen et al., 2003; Fravolini et al., 2018) e l'attività degli organismi decompositori (Bani et al, 2018; Pioli et al., 2018). Tuttavia, tra i vari fattori che influenzano il processo di decomposizione del legno morto, dalla letteratura scientifica non è ancora emerso con chiarezza quali possano essere quelli determinanti e di maggiore importanza (Cornwell et al., 2009; Freschet et al., 2012). La discussione sui fattori guida del processo di degradazione della necromassa risulta pertanto controversa, necessitando di ulteriori studi ed approfondimenti. Mentre numerosi studi hanno dimostrato che le caratteristiche del substrato legnoso (es. specie arborea, dimensione del substrato ecc.) siano determinanti nel processo di degradazione, altri sottolineano invece il ruolo delle condizioni climatiche (Bradford et al., 2014). In particolare, il clima, a livello locale e globale, rappresenta un fattore di fondamentale importanza nel processo di decomposizione del legno morto, in grado di influenzare l'abbondanza, la

diversificazione specifica e la conseguente attività degli organismi decompositori (Seibold et al., 2015; Hoppe et al., 2016). Tra gli organismi decompositori, conosciuti anche con il nome di saprofiti, cioè organismi che si nutrono di materia organica morta o in decomposizione, i funghi sono considerati i principali responsabili della degradazione della maggior parte dei materiali vegetali morti (Pioli et al., 2018). In particolare, tra questi i basidiomiceti sono noti per l'efficienza nel degradare il legno mediante la rapida depolimerizzazione delle componenti presenti nella parete cellulare delle piante, tra cui cellulosa, emicellulosa e lignina (Eriksson et al.,1990). In relazione alla componente della parete cellulare che attaccano e all'aspetto del legno in seguito alla degradazione, essi si dividono in funghi del marciume bruno, molle e bianco (Zabel and Morell, 1992). Tali funghi non colonizzano tuttavia il substrato legnoso contemporaneamente. Le specie fungine in grado di colonizzare il legno nelle prime fasi di degradazione prendono il nome di saprofiti primari. Si tratta di specie ruderali in grado di crescere velocemente sul legno con deboli capacità competitive nei confronti degli organismi antagonisti. Successivamente, tali organismi vengono sostituiti da specie fungine più resistenti, noti con il nome di saprofiti secondari (Owens et al., 1994). Durante le diverse fasi di decomposizione, le comunità fungine che si succedono sul substrato legnoso, grazie alla liberazione di diversi enzimi ligninolitici ossidativi, producono una serie di composti in parte derivanti dalla depolimerizzazione delle componenti della parete cellulare tra cui cellulosa, emicellulosa e lignina (es. zuccheri e fenoli semplici; Lynd et al., 2002; van den Brink and de Vries, 2011); ed altri prodotti durante la concorrenza con gli organismi antagonisti (Sonnenbichler et al., 1989; Hansson et al., 2012).

Tecniche analitiche per lo studio degli zuccheri e dei fenoli semplici prodotti in seguito alla degradazione del legno morto

In letteratura, sono riportati diversi metodi analitici per esaminare la composizione degli zuccheri (es. mono e disaccaridi) e dei fenoli semplici come prodotti di degradazione enzimatica della cellulosa, dell'emicellulosa e della lignina nel legno (Green et al., 1991; Pettersen, 1991; Medeiros et al.,2007; Abdelaziz et al., 2016). Tra i vari metodi utilizzati per l'analisi degli zuccheri troviamo quello calorimetrico, l'elettroforesi capillare, la gascromatografia (GC) e la cromatografia liquida (LC; Sluiter et al., 2010; Suksom et al., 2015). Tra questi, la GC e l'LC risultano quelli più utilizzati (De Goeij, 2013). Si tratta di due tecniche cromatografiche impiegate per la separazione e l'identificazione di due o più composti (es. zuccheri e fenoli) presenti in una miscela liquida o gassosa, che sfruttano l'equilibrio di affinità tra una "fase stazionaria" posta all'interno di una colonna cromatografica e una "fase mobile" gassosa nel caso della GC e liquida nel caso dell'LC che fluisce attraverso essa (De Goeij, 2013). Durante la separazione dei vari composti, quelli più affini alla fase stazionaria rispetto alla fase mobile impiegano un tempo maggiore a percorrere la colonna cromatografica (tempo di ritenzione), mentre i composti più affini alla fase mobile rispetto a quella stazionaria impiegano un tempo minore. Alla fine della colonna cromatografica è applicato un rilevatore (es. IR, UV-VIS, spettrometro di massa, PAD) che consente l'identificazione e la quantificazione dei vari composti tramite apposito cromatogramma (Swartz, 2010). Nonostante l'ampio utilizzo della GC e dell'LC per l'analisi degli zuccheri, entrambe le tecniche cromatografiche presentano alcuni svantaggi. A causa della bassa volatilità, dell'alta polarità ed idrofilia degli zuccheri, prima dell'analisi in GC tali composti devono essere convertiti in derivati volatili e stabili. Tale processo noto come derivatizzazione comporta un allungamento dei tempi di preparazione dei campioni da analizzare, ed il metodo GC risulta quindi poco pratico per effettuare le normali analisi di routine dei composti zuccherini (Sluiter et al., 2010; De Goeij, 2013). In cromatografia liquida, per consentire una separazione efficiente e selettiva degli zuccheri nei campioni da analizzare (es. legno) è necessario l'utilizzo di colonne cromatografiche specifiche (es. colonne di resina a scambio cationico o

stazionarie idrofiliche), in quanto quelle tradizionali a fase inversa risultano poco efficienti per la separazione cromatografica di tali analiti (Suksom et al., 2015). Negli ultimi anni, grazie allo sviluppo di fasi stazionarie (colonne cromatografiche) in grado di garantire un'adeguata separazione cromatografica dei composti (es. zuccheri) e all'alta sensibilità del rilevatore amperometrico pulsato (PAD), la cromatografia ionica (IC) accoppiata al rilevatore PAD è stata ampiamente utilizzata per l'analisi simultanea degli zuccheri in diverse matrici ambientali (Hoekman and Robbins, 2011; Widmer, 2011) tra cui il legno (Gamache et al., 2005; Raessler et al., 2010; Malacarne et al., 2016). Si tratta di una tecnica di cromatografia liquida ad elevate prestazioni che non prevede la derivatizzazione degli zuccheri durante le varie fasi preparatorie dei campioni, ottimizzando in questo modo i tempi richiesti per le analisi. Per la quantificazione di tutti gli zuccheri presenti all'interno del campione di legno mediante il rilevatore PAD è necessario effettuare diverse diluizioni dei campioni. Per evitare questo inconveniente, tale rilevatore può essere accoppiato ad un rilevatore ad aerosol (Corona CAD). Si tratta di un detector universale in grado di quantificare nell'ordine dei nanogrammi diversi composti organici indipendentemente dalla loro natura chimica tra cui gli zuccheri (Gamache et al., 2005).

Per quanto riguarda i composti fenolici, il loro rilevamento può essere effettuato mediante l'utilizzo di diverse tecniche analitiche tra cui: la gascromatografia (GC), la cromatografia liquida (LC), la cromatografia di esclusione (SEC), l'elettroforesi capillare (CE) e la cromatografia bidimensionale (2D; Abdelaziz et al., 2016). Tra le varie tecniche, la cromatografia liquida ad alte prestazioni (HPLC) accoppiata allo spettrometro di massa (MS) risulta molto efficace e selettiva per l'identificazione e la quantificazione dei composti fenolici quali prodotti di degradazione della lignina nel legno (Abdelaziz et al., 2016). L'HPLC-MS rispetto alla GC non richiede la derivatizzazione dei composti fenolici, i quali possono essere rapidamente rilevati e quantificati dallo spettrometro di massa mediante un cromatogramma (Barnaba et al., 2015).

Tale tecnica inoltre, presenta diversi vantaggi tra cui: l'utilizzo di colonne cromatografiche (fase stazionaria) di ridotte dimensioni che impediscono deviazioni e percorsi alternativi degli eluenti (fase

mobile), una velocità di eluizione costante e regolabile (passaggio della fase mobile attraverso la colonna), tempi di analisi ridotti e l'utilizzo di piccole quantità di campione per le analisi (nell'ordine dei 5-10 microgrammi) a favore di una maggiore accuratezza e precisione (Abdelaziz et al., 2016). Negli ultimi anni, una recente implementazione della tecnica LC-MS ha riguardato lo sviluppo della cromatografia liquida ad ultra alta prestazione accoppiata allo spettrometro di massa ibrido quadripolare orbitrap (LC-Q-Orbitrap; Barnaba et al., 2015). Si tratta di una tecnica cromatografica estremamente accurata e precisa per l'analisi di composti a basso peso molecolare (es. fenoli) che si avvale di colonne cromatografiche con diametro delle particelle molto minore oltre a pompe e parti meccaniche in grado di operare a pressioni di esercizio ancora più elevate rispetto alla tecnica LC-MS. Inoltre, la separazione cromatografica delle sostanze eluite risulta più efficiente ed avviene in tempi notevolmente ridotti. Infine, lo spettrometro di massa ibrido quadripolare orbitrap consente di ottenere dati ad alta risoluzione e accuratezza della massa (HRAM), generando misurazioni ad altissima risoluzione fino a 500.000 FWHM (Makarov et al., 2006; Makarov and Scigelova, 2010).

Obiettivi e struttura della tesi

La comprensione delle fluttuazioni della quantità e distribuzione del legno morto all'interno degli ecosistemi forestali in relazione a diversi fattori, come le pratiche di gestione selvicolturali ed i tassi di decomposizione, risulta di fondamentale importanza sia per la scelta di opzioni forestali sostenibili, volte a mitigare i cambiamenti climatici in corso e a preservare la biodiversità, sia per comprendere le dinamiche legate al ciclo dei nutrienti e del carbonio. In tale contesto si è inserita l'attività di ricerca svolta nel corso dei tre anni di dottorato.

Obiettivo principale del presente lavoro di tesi è stato quello di indagare i diversi fattori antropici (gestione forestale) e biologici (attività fungine) che influenzano l'abbondanza ed i processi di decomposizione del legno morto, con particolare riferimento a due specie forestali di ampia diffusione nei contesti forestali italiani ed europei: il faggio (*Fagus sylvatica* L.) e l'abete bianco (*Abies alba* Mill.). Sono state condotte tre attività di ricerca, connesse tra loro da un filo logico che ha tenuto conto di scale spaziali differenti. In dettaglio, il primo lavoro è stato realizzato nell'ambito del Progetto Internazionale LIFE+ ManforC.BD (*Managing forest for multiple purpose: carbon, biodiversity and socio - economic weelbing*) e descritto nel capitolo 1. Obiettivo di questo lavoro è stato quello di testare gli effetti a breve termine di pratiche selvicolturali innovative e tradizionali sulla presenza e distribuzione del legno morto (es. ceppaie, snag, alberi morti in piedi e detriti legnosi grossolani o CWD) e su altri indicatori di biodiversità (microhabitats e vegetazione del sottobosco) in tre faggete (*Fagus sylvatica* L.) montane dislocate lungo un transetto latitudinale in Italia. A partire da un approccio a scala di popolamento, che ha permesso di valutare la presenza del legno morto in relazione alla gestione forestale applicata, le attività di ricerca condotte nel corso del secondo e terzo anno di dottorato si sono concentrate su scale spaziali di maggior dettaglio. Esse hanno riguardato la caratterizzazione quali-quantitativa dei processi di decomposizione del legno morto, mediante analisi cromatografiche degli zuccheri e dei fenoli semplici in campioni di legno morto di abete bianco, spostando quindi l'approccio sperimentale in attività di laboratorio, gestite in condizioni controllate. Più in dettaglio, le attività descritte nel capitolo 2 hanno riguardato lo studio del profilo degli zuccheri

semplici quali prodotti di degradazione della componente polisaccaridica del legno (cellulosa ed emicellulosa), rilevati nel legno vivo di abete bianco (*Abies alba* Mill.) e nei campioni di CWD afferenti a cinque fasi di decadimento (secondo Hunter, 1990). Lo studio è stato condotto riferendosi a due periodi stagionali diversi ed in due siti forestali alpini situati nel Nord Italia (TN). In particolare, sono stati esaminati gli andamenti e la variabilità del contenuto degli zuccheri semplici durante il processo di decomposizione, utilizzando un cromatografo a scambio ionico, dotato di un rilevatore amperometrico pulsato ed un rilevatore ad aerosol (IC-PAD-CAD). In linea con quanto svolto nel secondo anno, le attività di ricerca sono proseguite con un ulteriore approfondimento dei processi di decomposizione del legno morto, analizzando i composti fenolici prodotti in seguito all'attività enzimatica spostando l'attenzione sull'azione dei funghi durante tale processo. In particolare, obiettivo dello studio descritto nel capitolo 3 è stato quello di analizzare l'andamento dei composti fenolici quali prodotti di degradazione della lignina nel legno di abete bianco prima e dopo l'azione enzimatica di nove funghi del marciume bianco appartenenti ai generi *Armillaria* spp. (*A. borealis, A. cepistipes, A. gallica, A. mellea, A. ostoyae, e A. tabescens*) ed *Heterobasidion spp*. (*H. abietinum, H. parviporum, H. annosum sensu stricto*). Tali funghi sono specializzati nei processi degradativi del legno di conifere, come ad esempio l'abete bianco (Capretti et al., 1990; Oliva et al., 2009). Le attività sono state condotte in condizioni controllate, in laboratorio, inoculando le nove specie fungine all'interno di vials contenenti segatura di abete bianco sterile e prelevando parte dei campioni in un intervallo temporale compreso tra i 2 ed i 6 mesi dall'inoculazione fungina. È stata quindi effettuata un'analisi dei composti fenolici presenti nei diversi campioni analizzati, utilizzando una tecnica cromatografica di recente innovazione e consistente in un cromatografo liquido ad alte prestazioni, accoppiato ad uno spettrometro di massa ibrido quadripolare orbitrap (LC-Q-Orbitrap).

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CAPITOLO 1

Early responses of biodiversity indicators to various thinning treatments in mountain beech forests

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Abstract

In recent decades, the conservation of biodiversity has become one of the main areas under consideration in managing forests in an ecologically sustainable way. Forest management practices are primary drivers of diversity and may enhance or decrease forest biodiversity, according to the measures applied (thinning options).

We have focused on three beech (*Fagus sylvatica* L.) forests across a latitudinal gradient in Italy, characterised by different structures resulting from dissimilar management. We tested the short-term effects of differently - based silvicultural intervention vs. stands where no silvicultural practices were applied on biodiversity indicators and related proxies: deadwood amounts, microhabitat density, floristic richness and life form abundance. In each study area, the occurrence of the cited indicators and proxies was evaluated before and after the implementation of crop tree thinning (CTT) and thinning from below (LT) methods, comparing them with control areas where no interventions were performed. After two years, the management options resulted in different responses of the investigated parameters. The CTT increased deadwood amounts in comparison with the LT ones, while stumps increased significantly after the LT thinning. Microhabitats increased significantly where intervention was not undertaken. On the contrary, they remained unvaried after the LT treatments. CTT thinning created favourable conditions for the development of microhabitats and their proliferation in the long term. Two years after the application of the CTT thinning treatment, all forest stands demonstrated a significant increase in their floristic richness and herb layer cover. Significant differences were also found in both the frequency and cover of life forms in relation to silvicultural treatment. These findings provide a better understanding of short-term effects of useful silvicultural treatment for maintaining biodiversity in mountain beech forests.

Keywords: Deadwood; Microhabitats; Understory vegetation; Mountain forests; Sustainable forest management; Italian forests.

1. Introduction

Forests are the most important global repositories of terrestrial biodiversity, which play an important role in functional ecosystems and the supply of a wide range of ecosystem services (Pecl et al. 2017). Climate and land use changes are threatening forest biodiversity, with negative effects on forest productivity and carbon uptake. The conservation of biodiversity is, therefore, a major goal when managing forests in a sustainable way (Lindenmayer et al. 2000). Sustainable forest management needs to integrate forest conservation practices based on multi-functional and flexible approaches (MacDicken et al. 2015), including potential values of climate regulation, habitat provision, water control, genetic resources, etc.. Indeed, forest management is an important factor that influences richness in species and ecosystem productivity and, in relation to the applied measures, may have positive or negative effects on forest biodiversity (Kutnar et al. 2015). Silvicultural strategies and conservation priorities focusing on site-specific guidelines for stand structures and harmonised use of the forest resources are recommended for achieving the required objectives in resilience and inclusiveness. Furthermore, they may involve emulating processes, which naturally occur in forest ecosystems (Gamborg & Larsen 2003).

When considering the multi-functionality of forest systems, different types of management can be applied to preserve forest functions and timber production in mountain environments. Sustainable forest management practices should be used to promote the conservation of biological forest resources on a global scale. At the local scale, forest management guidelines and forest certification programs may require maintaining or increasing the abundance of deadwood as an indicator of progress in maintaining biodiversity. Intensively managed forests show a reduction in the amount and variety of deadwood in comparison with unmanaged forests (Paillet et al. 2015), even though it is a key structural feature in forest ecosystems that supports species diversity (Burrascano et al. 2008, Lombardi et al. 2012).

Deadwood occurrence is essential for the maintenance of biodiversity, representing a source of nutrients for invertebrates (Siitonen 2001), bryophytes (Ódor & Standovár 2001) and lichens

(Humphrey et al. 2002). Deadwood substrates serve as a refuge that hosts a wide diversity of animals, such as amphibians (Herbeck & Larsen 1999), small mammals (Harmon et al. 1986), and birds (Mikusinski & Angelstam 1997). Deadwood amount continuously evolves in time, not only in relation to the intensity of silvicultural practices and the way they are carried out (Guby & Dobbertin 1996), but also due to the frequency, intensity and type of natural disturbances. Furthermore, the loss of microhabitats threatens biodiversity in forest ecosystems (Winter & Moller 2008). The term ‘‘microhabitat” encompasses several structural features on single trees and small substrates used by numerous species, or groups of species, to grow, nest or forage (Winter & Moller 2008). Microhabitats can be associated with decreasing tree vitality, which is commonly caused by a combination of fungi, viruses and bacteria (Larrieu & Cabanettes 2012). They are useful in describing the level of forest naturalness, integrating structural complexity and deadwood diversity (Michel & Winter 2009). Dying or dead trees offers a great variety of tree holes, which necessarily attract cavity nesting or roosting birds, and support large invertebrate communities (Brustel & Gouix 2011). Decaying deadwood provides nutrients and moisture that benefit many organisms, as well as shelter in extreme temperatures.

Understory vegetation is also a component of forest biodiversity and a key driver of many forest processes, including litter decomposition (Dearden & Wardle 2008), light interception (Gendron et al. 1998) and forest productivity (Nilsson & Wardle 2005). Understory vegetation constitutes the largest number of plant species that participate in forest dynamics. Single species or groups of species present on the forest floor can be used as proxies for site conditions (Khanina et al. 2007). Identifying the occurrence of plant species in the forest understory provides an assessment of the degree of naturalness, addressing suitable management models for the conservation of biodiversity (Nilsson & Wardle 2005). Forest management has a strong effect on floristic composition and, in particular, on the abundance of life form (Kern et al. 2014). The thinning activities, as well as natural disturbances, alter forest floor microclimatic conditions, promoting higher understory species densities (and deadwood decomposition rates), which in turn can affect other processes and taxa. However, our

understanding of how silvicultural treatments affect biodiversity indicators and how these effects interact with natural disturbances remains limited, particularly in the Mediterranean area.

The conservation of biodiversity in forests, therefore, requires investigation to define innovative silvicultural treatments that promote conservation purposes (Lindenmayer et al. 2000, Sculthe et al. 2006). In this study, we tested the short-term effects of differently-based silvicultural interventions (innovative and traditional) vs. stands where no silvicultural practices were applied on biodiversity indicators and related proxies. Specifically, we quantified the amount of deadwood, the density and variability of microhabitats, understory vegetation composition and the abundance of life forms before and after different types of forest thinning were applied to three mountain beech forests located along a latitudinal transect that stretches from southern to northern Italy. We hypothesised that the density of microhabitats, the amount of deadwood, the richness of species and the abundance of life forms would be reduced and simplified in relation to the intensity and type of applied silvicultural treatments.

2. Materials and methods

2.1 Study sites

The investigations was carried out in three study sites located across a latitudinal transect in Italian mountain beech forests (Figure 1), corresponding to different bioclimatic regions. In detail, they represent three different Habitats (*sensu* Habitats Directive 92/43 ECC): 9130 *Asperulo-Fagetum Fagus sylvatica* forests for the Alpine site, 9210 Apennine beech *Fagus sylvatica* forests with *Taxus* and *Ilex* for Central Apennine and 9220 Apennine *Fagus sylvatica* forests with *Abies alba* for Southern Apennine.

The northern study site of the transect is located in the *Cansiglio* area, in the Southern Alps (Veneto Region). The forest extends to 667 ha and is characterised by a high forest, where beech (*Fagus sylvatica* L.) is the dominant tree species. The forest consists of an even-aged (120 to 145 years) stand, where the applied silviculture consisted of low-mixed thinning repeated every 20-25 years on

the same management unit. The long-lasting repetition of silvicultural practices resulted in a fairly uniform stand structure (Becagli et al. 2013).

The *Chiarano-Sparvera* site is located in Central Italy, in the Apennine mountains (Abruzzo Region), included in the external protection zone of the "Abruzzo, Lazio and Molise" National Park. The total area extends to 766 ha, covered mainly by beech (95%). The principle historical management type is coppice with standards, nowadays characterised by an even-aged high forest, arising from a conversion treatment.

The *Mongiana* study site is located in the Southern Apennine (Calabria Region). The stand is a high forest dominated by beech that extends over an area of 1257 ha. Being located in the upper part of the mountain system facing the Tyrrhenian sea, its microclimate is influenced by the interception of fog, wet winds and precipitation (Becagli et al. 2013). The stand is an even-aged stand of about 70 years, where trees from the former cycles are present. The random occurrence of trees, which are much older than the dominant tree age makes the physiognomy less regular than a typical even-aged beech forest (Becagli et al. 2013).

Details on the main features of the study areas are reported in Table 1.

Table 1. Main geographic features of the three investigated sites located across a latitudinal transect in Italian mountain beech forests. Site 1 is located in the Southern Alps (Veneto); site 2 in Central Italy, on the Apennine mountains (Abruzzo), while the site 3 refers to the Southern Apennine (Calabria).

	Study sites		
	(1) Cansiglio	**(2) Chiarano-Sparvera**	**(3) Mongiana**
Area (ha)	~33	~30	~30
Coordinates (UTM-WGS84)	46°03' N 12° 23' E	41° 51' N 13° 57' E	38° 30' N 16° 14' E
Altitudinal range (m a.s.l)	1100-1200	1700-1800	~1100
Exposure	North	North-East	North-West
Bedrock	Limestone, Marlstone	Cretaceous limestone	Granite
Mean annual temperature (°C)	56	85	101
Annual precipitation (mm)	1660	1000	1880

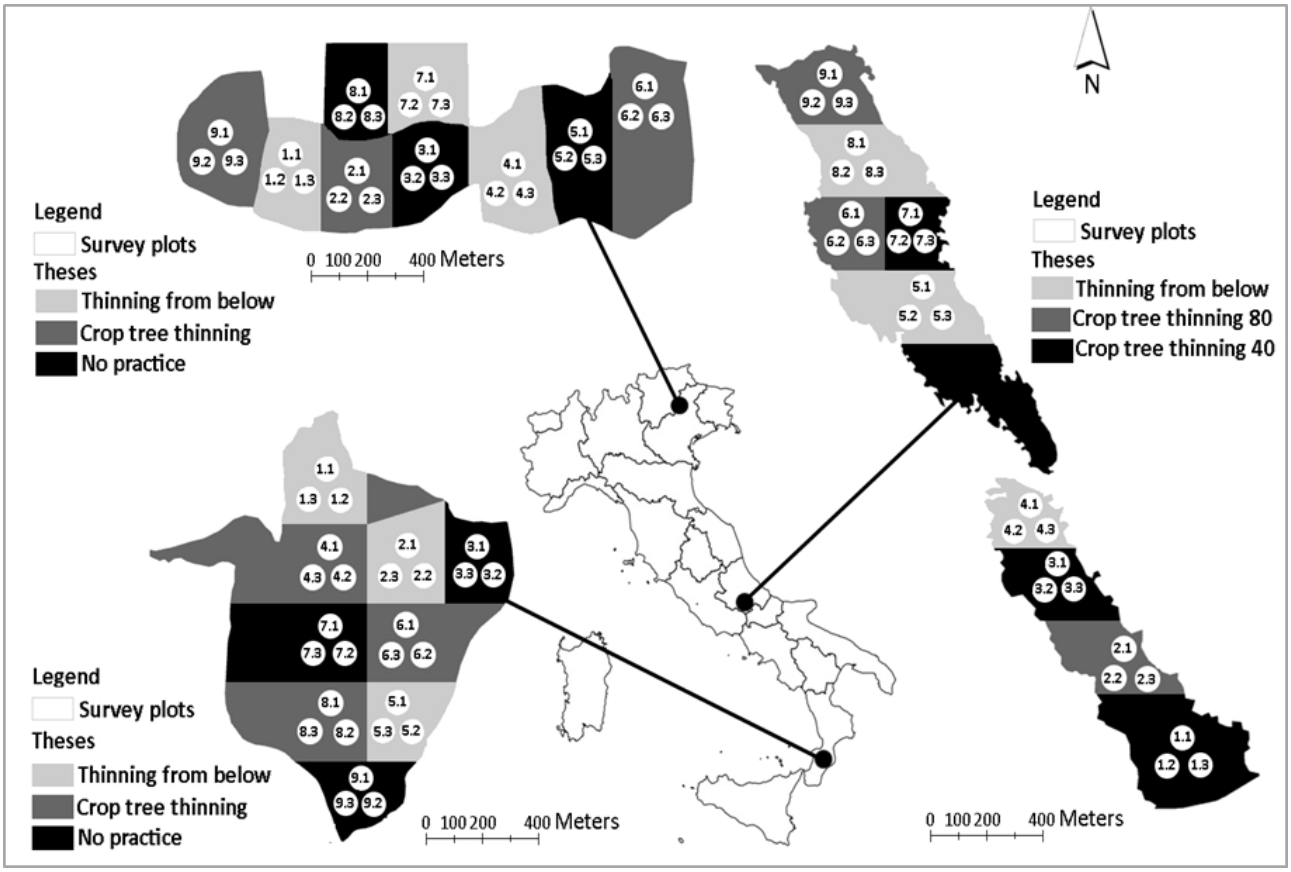
2.2 Survey scheme and types of treatments

Figure 1 reports the survey approach applied in the three study sites. An area of 30 hectares was devoted to the management trials at each site. Three silvicultural treatments (theses) were identified and randomly assigned in three replicates to each compartment. Each sector was defined using a 3-ha grid. In each sector, a cluster of three circular sampling plots (530 m^2) was established within each compartment, according to a systematic design (Becagli et al. 2013; Lombardi et al. 2015). A total of 81 plots were then sampled: 27 for each study area, 9 plots for each compartment (9 plots * 3 compartments * 3 study areas). For each study area, the traditional treatments were supported by one innovative silvicultural intervention, with the exception of Chiarano-Sparvera site, where two innovative interventions where applied. Traditional logging consisted in selective thinning (from below), reducing forest density through the removal of selected trees, but maintaining a homogeneous canopy cover. Innovative treatments consisted of opening canopy gaps (thinning from above), releasing candidate trees selected on the basis of their dominance and structure (shape, position, quality), and harvesting all adjacent trees.

In particular, one silvicultural treatment was characterised by the customary thinning practice, portrayed by a low-mixed thinning, as applied by local managers. The second silvicultural option consisted of an innovative criterion (I), concretised by a crown thinning at Cansiglio and in the selection of 45-50 trees per ha, with the removal of direct competitors at Mongiana. The no-thinning area ("No practice"), used as a control, integrated the experimental layout, but not at Chiarano-Sparvera, where two innovative theses were applied together with the LT treatment. In detail, the CTT treatments used were the "CTT 40" and the "CTT 80", consisting of a selective thinning, which was applied to support the best 40 trees per hectare and the best 80 trees per hectare, respectively. For further details on the differences between the LT and CTT silvicultural approaches and related harvested forest biomass, see Di Salvatore et al. (2016). Additionally, deadwood was artificially enriched near the ground in all the "innovative" treatments, leaving both coarse woody debris and dead trees lying on the forest floor. All wood was taken from freshly felled trees. Moreover, 2-3 living

stems per hectare were girdled to create standing dead trees, or felled and left on the ground to establish microhabitats and corridors for saproxylic insects and micro-fauna. In order to test the effectiveness of management options, the biodiversity indicators and proxies (deadwood, microhabitats and understory vegetation) were sampled before and after the silvicultural interventions. More specifically, the effects of the thinning intervention were tested two years after the silvicultural treatments were performed.

Fig. 1. Location of the three study sites and the survey protocol applied in Cansiglio (a), Chiarano-Sparvera (b) and Mongiana (c).



2.3 Deadwood and microhabitats

In each plot, deadwood was assessed according to the following parameters: standing dead trees, downed dead trees, coarse woody debris (CWD) and stumps (Table 2).

Standing dead trees, downed dead trees and stumps were included in the survey, when more than half of the base of the trunk lay within the plot. Coarse woody pieces were included when more than half of the base of the thicker end lay within the plot. A threshold height of 1.3 m was used to distinguish stumps (less than 1.3 m) from standing dead trees (higher than 1.3 m) (Lombardi et al. 2012).

The volume of standing and downed dead trees was calculated using the equations derived for the prediction of the aboveground tree volume (Tabacchi et al. 2011). Moreover, the volume of stumps and coarse woody debris was calculated using the following equation (1):

$$(1)\ V = \tfrac{1}{3}\pi h(R^2+rR+r^2)$$

where: V = volume (m^3); h = height or length (m); R = maximum radius (m); r = minimum radius (m).

Volumes in m^3 were converted into volumes per hectare by applying an expansion factor, they were then grouped in relation to the different silvicultural treatments.

Moreover, excluding the deadwood, a set of twenty-three microhabitats was measured for each plot, following the survey scheme used by Winter & Moller (2008), before and after the silvicultural interventions.

We surveyed whole standing dead and living trees and deadwood components when their diameter was > 5 cm, carefully examining the trunk from the ground to the crown or the entire length of horizontal deadwood elements. The three observers for all plots used the same sampling approach both before and after the silvicultural treatments. Microhabitats were counted on each dead and living forest component, then converted into total numbers per hectare.

Table 2. Deadwood attributes sampled before and after the thinning operations. A total of 81 plots were realized: 27 for each study area, 9 plot for each compartment (9 plots * 3 compartments * 3

study areas) Abbreviations are: dbh130: diameter at breast height; H: height; L: length; Dmin: minimum diameter; Dmax: maximum diameter; Dbase: diameter at the base of the trunk; Dtop: diameter at the top of the trunk.

Deadwood component	**Dimensional limits**	**Parameters recorded**
Dead downed trees	$dbh_{130} \geq 5cm$, $L \geq 130cm$	Species, dbh_{130}, L
Standing dead trees	$dbh_{130} \geq 5cm$, $H > 130cm$	Species, dbh_{130}, H
CWD (Coarse Woody Debris)	$D_{min} \geq 5cm$, $L \geq 100cm$	Species, $D_{min,}$ D_{max}, L
Stumps	$D_{top} \geq 5cm$, $H \leq 130cm$	Species, D_{base}, D_{top}, H

2.4 Understory vegetation assessment

Understory vegetation was assessed before and two years after the implementation of the silvicultural treatments. The sampling unit consisted of a 400 m^2 square plot, centred in the plots where deadwood and microhabitats had provided samples. Within the plot, the percentage cover of each vegetation layer (tree, shrub and herbaceous layer), vascular flora and the percentage cover for each species were recorded by visual estimate. Furthermore, topographic features; such as elevation, aspect and slope, were also recorded. Those species detected were identified using the analytic keys of Italian Flora (Pignatti 1982). The vascular plant species list and relative percentage cover recorded in each plot were structured into five macro-categories of life form: chamephytes (Ch), geophytes (G), hemicryptophytes, (H) phanerophytes (P) and terophytes (T), according to Christen Raunkiaer's classification. Finally, the cover of herbaceous layer, species richness (number of species), frequency and cover for each life-form group were calculated for each plot and at site level, before and after the thinning interventions.

2.5 Statistical approach

In order to evaluate the effects of the silvicultural treatments for each geographical area investigated, the amounts of deadwood and microhabitats obtained before (*ex-ante*) and after (*ex-post*) the intervention phases were compared using IBM SPSS Statistics software, version 20.0 (Armonk, NY: IBM Corp.). The herb layer cover, the floristic richness and the biological spectrum relating to frequency and species coverage were also assessed, at site level, both before and after the silvicultural treatments.

The distribution of each population was tested using the Shapiro-Wilk normality-test. If the result of the normality test was positive, parametric comparison methods were adopted; otherwise, non-parametric comparison tests were assumed. Post-hoc mean comparison tests were performed via the Tuckey HSD test, when statistically significant differences between group means were determined by one-way ANOVA. When variables had non-Gaussian distribution and the non-parametric

Kruskal-Wallis test revealed differences among populations, post-hoc comparisons were performed through Dunn's test.

3. Results

3.1 Deadwood amounts

We observed different results related to the silvicultural treatments. Data collected before the treatments showed a high variability in volume among the different deadwood components (Figure 2). After the implementation of the silvicultural treatments, deadwood volumes revealed differences among the study sites.

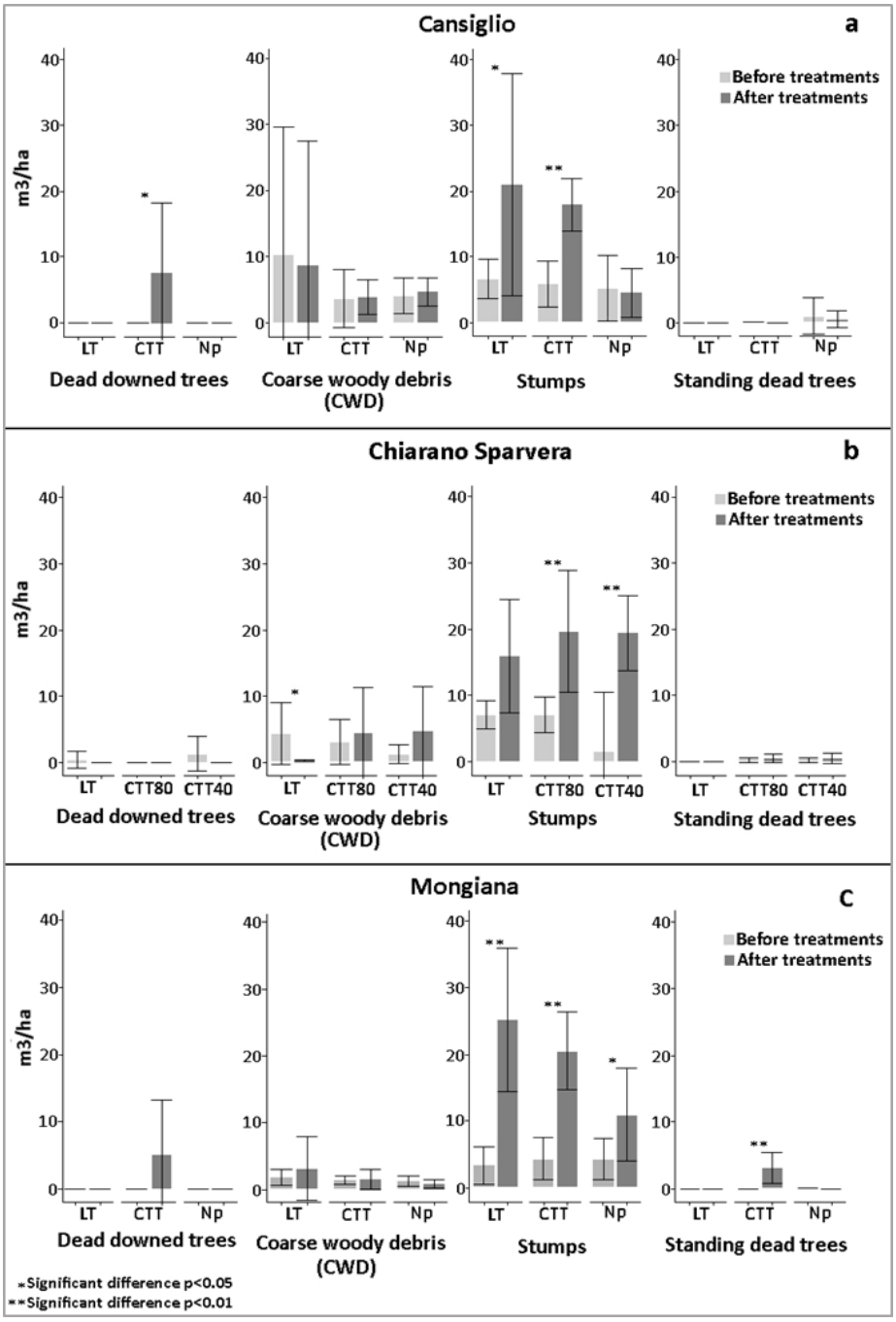
In Cansiglio (Figure 2a), stumps ranged between 17.94 and 21.05 m^3 ha^{-1} in the CTT and LT treatments, respectively. The volume of stumps was significantly higher following the CTT and LT treatments ($p<0.01$ and $p<0.05$), while it did not vary for the control without interventions ("No Practice" treatment). Moreover, volumes of downed dead trees significantly increased to 7.60 m^3 ha^{-1} after the CTT treatment ($p<0.01$).

In Chiarano-Sparvera (Figure 2b), results revealed a scarce presence of downed dead trees, both before and after the treatments, with a total absence in the CTT80 treatment. Before the silvicultural treatments, coarse woody debris volume showed a mean of only 5.5 m^3 ha^{-1}. Silvicultural treatments lead to a drastic reduction of coarse woody debris after the low thinning approach, with a significant decrease ($p<0.05$) of 4.60 m^3 ha^{-1}. In the other two sites, this result after this treatment was not observed. The volume of stumps did not increase after the treatment, while it was significantly higher for both the CTT40 ($p<0.01$) and CTT80 ($p<0.01$) treatments. The amount of standing dead trees was low before the silvicultural treatments and did not vary significantly thereafter.

All the silvicultural treatments applied in Mongiana lead to a significant increase in the volume of stumps (Figure 2c). In detail, a significant increase ($p<0.01$) of about 15 m^3 ha^{-1} was observed after the innovative treatment; in the "No practice" area, increases of nearly 20 m^3 ha^{-1} ($p<0.01$) and 5 ($p<0.05$) m^3 ha^{-1} were observed, respectively. The volume of standing dead trees increased significantly to about 3 m^3 ha^{-1} ($p<0.01$) only after the innovative treatment. The volume of downed

dead trees increased to 5 m^3 ha^{-1} after the innovative treatment, although the increase was not statistically significant.

Fig. 2. Amount (m3 ha-1) of deadwood components before and after the cutting activities in Cansiglio (a), Chiarano-Sparvera (b) and Mongiana (c) with related statistical comparisons *($p<0.05$) and ** ($p<0.01$).



3.2 Microhabitats

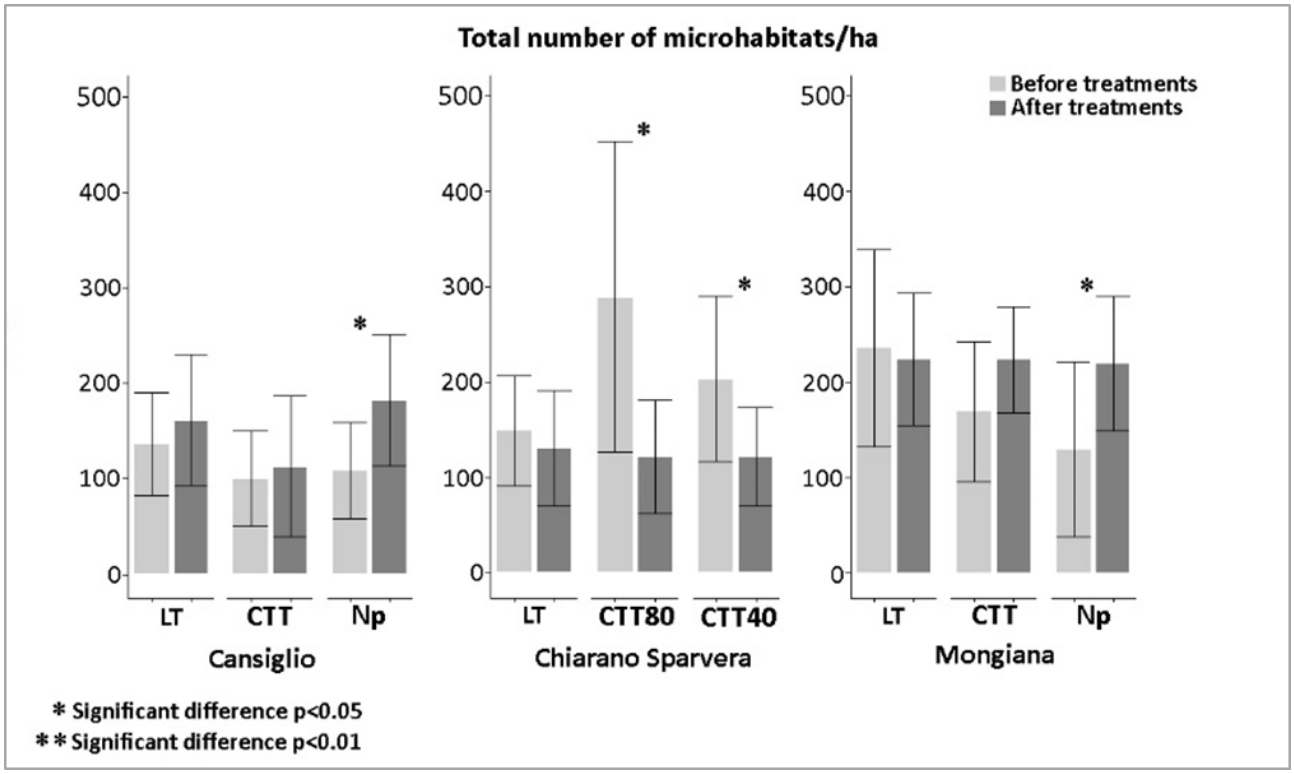
In general, a high variability in the number of microhabitats per hectare was observed in the study sites, although less evidently for Cansiglio, both before and after the silvicultural treatments (Figure 3). No significant differences were observed after the low thinning treatment for all three study sites. Both before and after silvicultural treatments, the total number of microhabitats was around 150 elements per hectare in Cansiglio and Chiarano-Sparvera, while this value was higher in Mongiana; about 230 microhabitats per hectare.

On the contrary, in Cansiglio and Mongiana, a significant increase in the number of microhabitats was observed, where the silvicultural treatments were not undertaken. Particularly, in Cansiglio, the number of microhabitats increased ($p<0.05$) to about 70 units per hectare, while in Mongiana, the number of microhabitats reached nearly 220 units per hectare, with an increase of about 90 microhabitats ($p<0.05$).

Thinning from above did not produce a significant increase in the number of microhabitats after the innovative treatment in Cansiglio, while a significant decrease ($p<0.05$) in the number of microhabitats was observed in Chiarano-Sparvera following both innovative treatments.

In particular, a marked decrease from 300 to less than 130 microhabitats per hectare was observed after the CTT80 treatment

Fig. 3. Total density (N/ha) of microhabitats and statistical differences (*p<0.05; ** p<0.01) in the three sites, before and after silvicultural intervention.



3.3 Species richness and cover

Before the implementation of the silvicultural treatments, a total of 178 plant species were recorded in all vegetation plots. In detail, comparing the three sites, different effects were observed, particularly concerning the type of treatments and species richness (Figure 4).

In Cansiglio, the innovative treatments lead to a significant increase ($p<0.05$) in species richness with a mean of about 26, while in Mongiana the increase was marginal. In Chiarano-Sparvera, the increase of species richness was significant after all silvicultural treatments. In detail, significant increases ($p<0.01$) with a mean of about 20.1 and 22.1 were observed after the low thinning and CTT40 treatments, while a significant increase ($p<0.05$) with a mean of about 17.6 was recorded in the I80 treatment. Significant increases in plant cover species were recorded in all sites with a medium increment of 55% (Figure 5). The total plant cover is often higher than 100% since all the vegetation layers, which frequently overlap, were considered as the sum of the cover values for each sampled species/layer. In particular, in Chiarano-Sparvera, plant species-cover increased significantly ($p<0.01$) after all silvicultural treatments. In Cansiglio, a significant increase was observed after both low thinning ($p<0.01$) and crop tree thinning ($p<0.05$) treatments. By contrast, in Mongiana, a significant increase in plant species-cover was observed after low thinning, innovative and "No practice" treatments ($p<0.05$).

Fig. 4. Number of species recorded before and after the silvicultural treatment in the three study sites, with related statistical comparisons *(p<0.05) and ** (p<0.01).

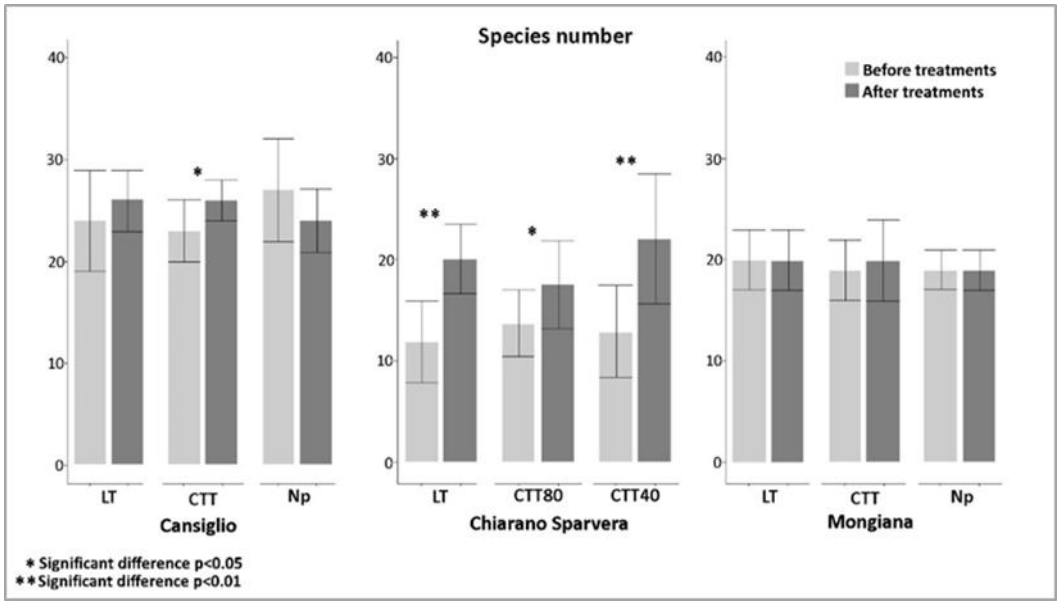
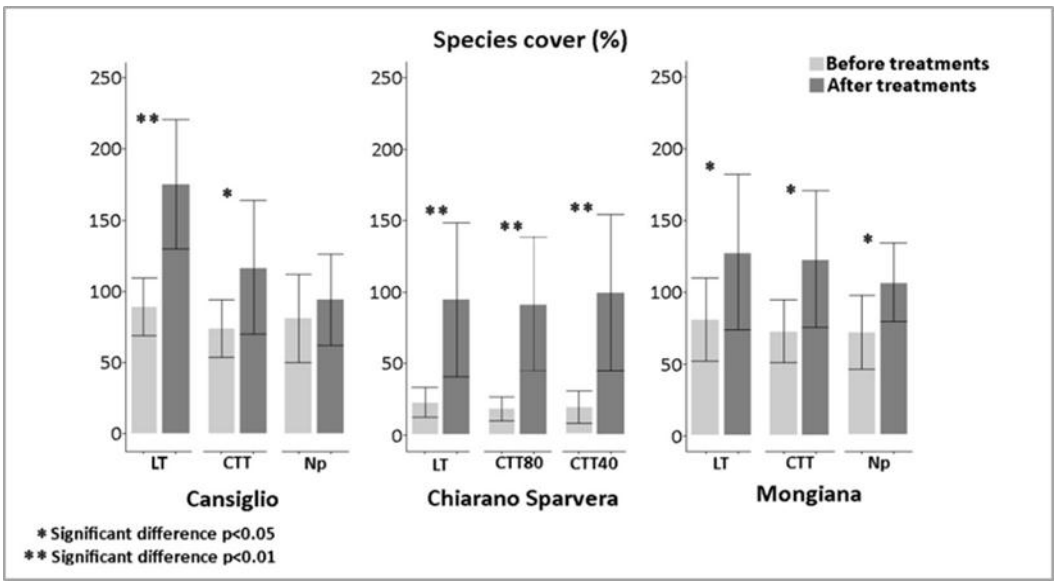


Fig. 5. Cover (%) of plant species observed before and after the silvicultural treatment in the three study sites, with related statistical comparisons *(p<0.05) and ** (p<0.01). Plant cover is often higher than 100%, since the sum of the cover values for the different layers (frequently overlapped) was considered.



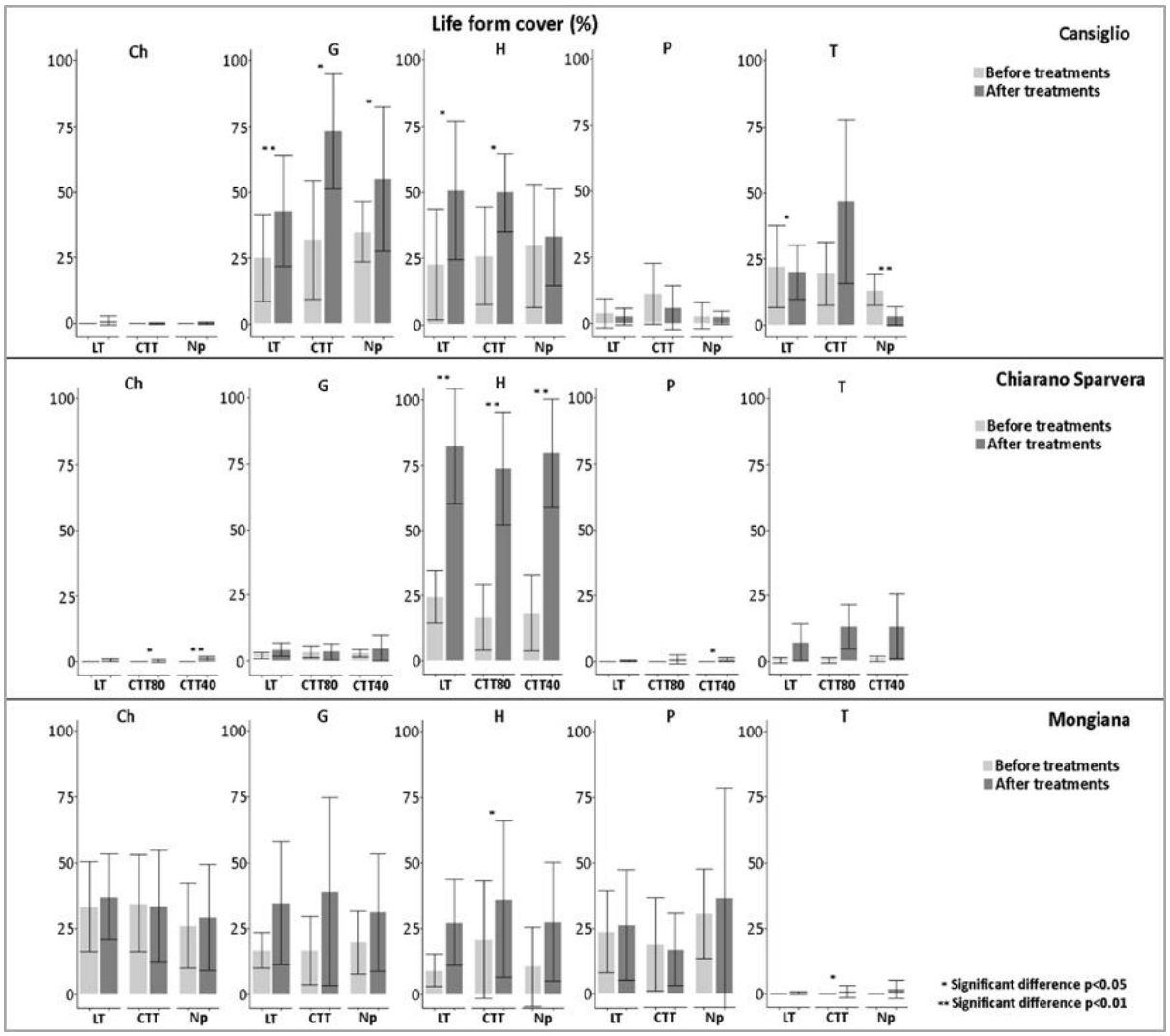
3.4 Life forms

Two years after the silvicultural treatment, significant differences were found in the frequency of life forms for the three studied sites (data not shown).

In all sites, after innovative treatment the frequency of therophytes was higher ($p<0.01$) than other life forms. The majority of these species appeared in the canopy gaps. In Cansiglio, a higher frequency of geophytes and lower frequency of hemicryptophytes were detected in the control plots. In Chiarano-Sparvera, hemicryptophytes increased after CTT80 (on average by 12.78%) and CTT40 (on average by 15.89%) treatments, with a decrease of geophytes after all the silvicultural treatment: low thinning (on average by 1.56%), CTT80 (on average by 1.89%) and CTT40 (on average by 2%). Finally, in Mongiana, the frequency of hemicryptophytes increased significantly ($p<0.01$) only after the innovative treatment.

After the silvicultural treatment, significant changes also affected the total cover of each life form group (Figure 6). In all sites, the cover of hemicryptophytes increased after innovative treatment. This increase particularly concerned Chiarano-Sparvera ($p<0.01$) after all the silvicultural treatment. In Cansiglio, the cover of geophytes reached high values after all the silvicultural treatment, while the cover of therophytes was low in the control plots (on average by 3.37%). In Chiarano-Sparvera, in particular, after the CTT40 treatment, a significant increase in cover of phanerophytes ($p<0.05$) and chamaephytes ($p<0.01$) was observed; this latter biological group increased ($p<0.05$) also after the CTT80 treatment. In Mongiana, after innovative treatment, an increase in cover of hemicryptophytes and terophytes was recorded ($p<0.05$).

Fig. 6. Cover (%) of life forms recorded before and after the silvicultural treatment in the three study sites, with related statistical comparisons *($p<0.05$) and **($p<0.01$). Ch, chamephytes; G, geophytes; H, hemicryptophytes; P, phanerophytes; T, terophytes; CTT, Crop Tree Thinning ; LT, Thinning From Below; Np, No practices.



4. Discussion

4.1 *Deadwood and microhabitats*

The main outcome of this work was that the early responses of Italian mountain beech forests to innovative silvicultural treatment were related to the increase and diversification of deadwood, and the creation of favourable conditions for the establishment of new microhabitats. Several authors have emphasised that silvicultural treatment should mimic natural disturbance (Haila et al. 1994) and highlighted the need for innovative management measures to conserve biodiversity and increase naturalness. We propose crop tree thinning, selective thinning from above, releasing a variable number of candidate trees, as innovative and diversification silvicultural practices aimed at increasing deadwood quantity and microhabitat density in these mountain beech forests, in order to combine forest productivity and carbon storage, and promote forest biodiversity. In particular, the innovativeness of the method lies in the number of candidate trees, which is rather low, since only 40-50 or 80 trees were selected.

In all experimental sites, local traditions and past habits influenced the impact of silvicultural treatment to a variable extent, further diversifying deadwood composition, in particular that of coarse woody debris and stump components. As regards the stumps, it is worth considering that our findings refer to the moment shortly after the application of silvicultural treatment (Harmon et al. 1986). Therefore, it is possible that some of these stumps are still able to re-sprout, and their number was overestimated. One area of innovative treatment; the cutting and releasing of several medium sized trees onto the forest floor, as future decaying deadwood, favoured the increase of coarse debris occurrence (Rouvinen & Kuuvalainen 2001), although the high standard deviation highlighted an irregular distribution of downed dead trees.

The integration of other practices, in addition to leaving the felled trees *in situ*, may help promote biodiversity. Indeed, in the long term, the girdling of trees during the thinning operations may increase the number of standing dead trees and deadwood occurrence, favouring a greater diversity of flora and fauna (Kerr 1999). Other studies have questioned the increasing occurrence of deadwood after

cutting, suggesting that deadwood fraction initially declines in a felled forest, due to its post-harvesting removal, but returns to pre-logging values after about 50 years (Borman & Likens 2012). Although partial harvests generally cause minor disturbances to ecosystem functions and services, the long-term impact of even light partial harvesting on coarse woody debris stocks and distribution can be observed (Morrissey et al. 2014).

In this study, we found, on average, 17 m^3 ha^{-1} of deadwood; 75% of this value is composed of stumps. Stumps, in fact, are directly related to management practices, as they are generally harvesting residuals (Burrascano et al. 2008). However, it is not obvious that the volume of stumps were significantly higher following the crop tree thinning and low thinning treatments, while they did not vary for the control without intervention. In fact, natural disturbances play their part in tree fall and thus in the creation of new stumps of natural origin, also in unmanaged stands (Lombardi et al. 2008a). In beech forests of the central Apennines, Lombardi et al. (2008b) the amount of deadwood ranges from 25 m^3 ha^{-1} in unmanaged stands to 5 m^3 ha^{-1} in managed ones, in line with our results. On average, the Italian National Forest Inventory estimates deadwood occurrence in Italy equal to 8.8 m^3 ha^{-1}. Mediterranean forests have a recent history of sustainable forest management practices, with deadwood input so far being limited to small, rapidly decaying material. When low thinning treatment was applied, the coarse woody debris component decreased significantly and downed dead trees disappeared. In these forests, lying deadwood was traditionally removed as firewood and to reduce the presence of physical obstacles to forestry operations (Pedlar et al. 2002).

Due to the absence of human pressure and thanks to the presence of old trees, an increase of some microhabitats was observed, only a short time after treatment; in particular fungal infections, cracks, cavities, bark loss, and woody debris with saproxylic insect holes. Beside deadwood occurrence, monitoring microhabitats in forest stands was an important option for validating the presence of a wide range organisms, since their incidence is strongly related to forest species (Harmon et al. 1986, Remm et al. 2006), as well as the efficacy of silvicultural treatments in the maintenance of forest biodiversity. However, their relationship to microhabitats with silvicultural treatments was different

from that of the deadwood. In general, thinning from below did not produce any marked decrease of microhabitats, and therefore, that method can be considered sustainable as thinning from above for preserving biodiversity in managed beech forests. Notwithstanding this innovative treatment aimed at increasing the occurrence of microhabitats, this was not the case in the studied forest stands. Innovative treatments, CTT40 and CTT80, even caused a significant decrease in the number of microhabitats. It must be pointed out that, due to the time needed for the establishment and diversification of microhabitats, these results may vary in a long-term perspective. For example, Larrieu et al. (2017) have observed significantly higher microhabitat density and diversity in beech forest stands harvested 10-15 years before surveying, while no differences in areas harvested 1-5 years before surveying, with marked differences occurring only 70-80 years after the last silvicultural intervention. Vuidot et al. (2011) have demonstrated that, whatever form of management, the number and type of microhabitats are mainly influenced by individual tree features, and the probability of microhabitat density is the same for similar trees.

In addition to forest operations, deadwood presence can also be linked to a major recurrence of natural disturbances (wind, fungal infections, etc.) and the consequent mortality of trees, which in turn may increase the accumulation of deadwood and microhabitat density, with further implications on these forest types. The implementation of innovative treatment, which emulate natural forest disturbance (opening of canopy gaps), represents a valid alternative, at least for the investigated forest types, to establish the practical environmental conditions to preserve or maybe increase, over a long term prospective, the levels of forest naturalness and sustainability.

4.2 Understory vegetation and life forms

In the Cansiglio and Chiarano-Sparvera sites, the floristic richness was only higher in the areas affected by innovative treatment. The intensity of this treatment induced a significant decrease in tree density, creating gaps and increasing the input of light radiation, useful to the rapid colonisation of gaps (Kutnar et al. 2015). Within forest canopy gaps, the availability of nutrients to understory vegetation can be higher, due to limited competition and increased insolation and temperature (Fahey

& Puettmann 2007), although in the Mediterranean environment an increase in water loss can also be expected, due to higher evaporation rates. In Mongiana, significant differences were not found in the number of species after the application of silvicultural treatments. Studies evaluating selective logging and thinning treatments also showed inconsistent effects on understory vegetation richness (Paillet et al. 2010). These results can be influenced by the forest type examined. In fact, in Mongiana, the higher heterogeneity of forest stands observed before the implementation of silvicultural treatment did not implicate significant changes in tree density, so that the species richness was not affected.

Even if species richness and forest management are strongly interconnected, a few studies have been conducted on the responses of the understory vegetation to the thinning activities. Forest management includes various methods of logging, which can differentially influence the understory vegetation. For example, a peculiar cutting scheme may affect plant cover differently from species richness (Knapp et al. 2013). In this study, the implementation of different silvicultural treatments (thinning intensity and type) caused specific impact on the composition of understory vegetation.

Several studies have shown that floristic richness is inversely proportional to tree density (Götmark et al. 2005). In addition to species richness, significant differences were observed by analysing the cover values of herbaceous species in relation to the type of silvicultural treatment. In Cansiglio and Chiarano-Sparvera, plant cover was higher in the areas where both thinning from below and crop tree thinning were implemented. In these areas, a rapid colonisation of gaps was recorded and, besides the nemoral species, forest edge species also appeared in the mixed understory vegetation. Furthermore, at Mongiana site, a significant increase in understory plant cover was observed, also in the control plots. This increase can probably be influenced by the specific microclimate of this Mediterranean mountain forest stand, where herbaceous species have adapted to shady and humid conditions and may find optimal growth environments.

Moreover, concerning life forms, different trends among the three sites were observed. In Cansiglio, a significant increase in the frequency of geophytes was observed in the control plots, while the cover of these species (e.g. *Anemone nemorosa* L., *Anemone trifolia* L.) increased in all three types of

silvicultural treatment. In nearby Alpine forests, Alberti et al. (2013) found that the abundance of geophytes groups is strictly related to the degree of shading of the forest stand (e.g. tree density, canopy cover). In our site, the frequency of hemicryptophytes decreased in the control plots, while the cover of these species increased after thinning from below and crop tree thinning treatment. Biswas & Mallik (2010) found a significant increase in graminoids (hemicryptophytes), but also a significant decrease in tree species, with increasing disturbance intensity. The consequences of disturbances on biodiversity are complex and are influenced by disturbance characteristics (type, intensity, etc.) and community traits (composition, richness, etc.). The consequences of severe disturbances may even be negligible on understory individuals if, for example, increased mortality rates of canopy trees are accompanied by an increase in available resources (e.g. nutrients, light, etc.) and there are available recruits. Increasing overall species richness and decreasing total ecosystem carbon can be expected under a climate change scenario (Thom & Seidl 2016). Yet, in European forests, the nature and regime of disturbances have changed through time, and sustainable silvicultural practices need to take natural disturbance dynamics into account to better manage forests for both production and biodiversity.

The frequency of therophytes increased after crop tree thinning treatment, but decreased in the control plots, probably because of the continuity of the forest cover. Graae & Sunde (2000) have reported that Raunkiaers therophytes are more common in managed than in unmanaged forests. In Chiarano-Sparvera, the dominance of hemicryptophytes was higher after CTT80 and CTT40 treatments than in the control plots; whereas, the occurrence of geophytes was lower after treatment in all the study sites. The reduction of tree cover and the creation of canopy gaps increased the light radiation on the forest floor, in turn reducing the frequency of geophytes. Moreover, after CTT80 and CTT40 treatments, the high frequency of hemicryptophytes was strictly connected to the establishment of species typical to grazed areas, surrounding the studied forests. Among the typical species of pastures, there were *Dactylis glomerata* L., *Cirsium tenoreanum* Petr., *Capsella bursa-pastoris* (L.) Medik. These species generally take advantage from disturbances, either natural or anthropogenic, due to gap opening

processes. In Chiarano-Sparvera, a significant increase in the cover of chamaephytes was recorded after both the CTT80 and CTT40 treatment. This increase was related to the presence of *Trifolium pratense* L., which was not detected before the implementation of the silvicultural treatment. This species is commonly found in nearby meadows and pastures. In addition, in CTT40 areas, an increase in the cover of phanerophytes was also found, as a further consequence of thinning practices. In Mongiana, after the crop tree thinning treatment, higher frequency and coverage of therophytes and hemicryptophytes were observed. Hodgson et al. (2005) observed that annual species (therophytes) and graminoids (hemicryptophytes) are dominant in disturbed habitats, but also where trees are prevalent.

5. Conclusions

This study confirms the importance of deadwood, microhabitats and understory vegetation as biodiversity indicators and proxies, when related to silvicultural strategies for mountain beech forests. After two years from treatment, the management options lead to different early responses of the studied parameters. Crop tree thinning treatments increased deadwood quantities, while thinning from below favoured only the presence of stumps. Microhabitats increased in number where silvicultural intervention was postponed and remained unvaried after the LT intervention. CTT treatment created ecological conditions, which were beneficial to the establishment of microhabitats and their development in the future. Furthermore, a significant increase in floristic richness was observed after CTT treatment, in all the studied forest stands. Finally, further monitoring activities in the same study areas will be desirable in the next 5-10 years, to further investigate the timber quality after thinning at the rotation age.

The results point to adaptive forest management strategies leading to sustainable utilisation rates that allow for maintaining forest productivity and increasing biodiversity conservation. Although most studies of biodiversity-oriented management strategies focus on forest biodiversity indicators, which are separate from each other, integrating multiple indicators proved more practical in targeting sustainable silvicultural treatment and its associated functions.

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CAPITOLO 2

CHARACTERISATION OF SILVER FIR WOOD DECAY CLASSES USING SUGAR METABOLITES DETECTED WITH ION CHROMATOGRAPHY (IC).

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ABSTRACT

Hunter's classification is the most adopted method for describing coarse woody debris (CWD) decay stages; however, wood chemical characterization is often lacking. This study explores the sugar profiles of living silver fir (*Abies alba* Mill.) trees and CWD samples in Hunter's five decay stages, evaluating the trends and variability in sugar content in CWD samples during decomposition.

Wood cores from living trees and CWD samples from 2 different forest sites in Trentino (Italy) were analysed after extraction using ion-exchange chromatography, equipped with pulsed amperometric

and charge aerosol detectors (IC-PAD-CAD). Detailed monosaccharides, disaccharides and sugar alcohols profiles of living wood and CWD samples were described. Cellobiose, arabinose, maltose and trehalose showed similar trend in both the sites. Principal Component Analysis (PCA) highlighted the good capability of sugar profiles to well characterise and discriminate silver fir wood samples among Hunter's stages.

Keywords: deadwood, cellulose, sugars, IC-PAD-CAD, decomposition stages.

INTRODUCTION

Coarse woody debris (CWD) is the residue of living trees, including whole fallen trees, fallen branches, pieces of fragmented wood, stumps, standing dead trees and snags [1, 2]. Nowadays, the increasing attention to CWD function and decomposition makes the management of deadwood in forest a further objective, providing a chance to integrate many related research items [3]. CWD is an important component in the functioning of forest ecosystems, influencing nutrient cycling [4], humus formation and carbon storage [5]. CWD biomass varies from 10% to more than 30%, in relation to forest types [6-7] and stand management [3]. As a consequence of global warming, a decrease in CWD could be expected, due to enhanced decomposition rates [8]. In temperate Europe, studies on CWD decay progression have been, however, scarce and poorly described in comparison to other ecosystem processes. Nutrient cycling during CWD decay progression varies in relation to the different tree species and geographical context [9], and a change in biogeochemical cycles could be also expected, due to warming temperature.

Recent comparisons of tree species suggest that silver fir (*Abies alba* Mill.) can be considered particularly susceptible to climate change [10-11]. Understanding fluctuations in CWD abundance and decomposition rates is very important in order to analyse forest and carbon dynamics [12]. In the literature, several ways to understand and describe CWD-related parameters have been reported. In the main European and American forest inventories, the most widely adopted classification of CWD

is a five decay-class system developed by Hunter [13-15]. This system is based on visual, geometric and tactile characteristics of wood in the field (Table 1).

Table 1: The five CWD decay classes according to Hunter's classification (1990).

CWD features	Decay classes				
	Class 1	**Class 2**	**Class 3**	**Class 4**	**Class 5**
Bark	Intact	Partially absent	Almost completely absent	Absent	Absent
Branches	Present	Absent	Absent	Absent	Absent
Wood texture	Intact	From intact to a little soft	Soft outer layer, intact inner part	Small pieces, soft	Very soft (powdery or fibrous)
Wood shape	Round	Round	Round	From round to oval	Oval
Wood colour	Unaltered colour	Unaltered colour	Colour a little faded	Colour light/ brown	Colour light / yellow or gray
Contact with soil	Log elevated on what remains of branches	Log Partially resting on the soil	Log for the most part resting on the soil	Log in contact with soil	Log in contact with soil and partially buried

Unfortunately, Hunter's classification is based on purely macromorphological observations, and several authors have suggested that wood chemical investigation (*e.g.* wood density, carbon, cellulose determination) is necessary to better assess the different decay stages of CWD [5, 16-18]. Cellulose is the main polysaccharide found in both the primary and secondary cell walls, constituting up to 50% of mass in trees. The chemical composition is simple, consisting of several hundred to many thousands of D-glucose units, linked by β-1,4-glucosidic bonds. Cellulose chains, which are held together by intermolecular hydrogen bonds create linear crystalline structures (microfibrils) and less crystalline, (amorphous regions). The ratio between these regions varies in relation to the layer of primary and secondary cell walls and between different plant species. In the plant cell wall, the cellulose microfibrils are embedded, mainly with hemicellulose, pectic polysaccharides and lignin [19, 20]. Hemicellulose, is a heteropolymers of anhydro-sugar units linked by glycosidic bonds. Unlike cellulose, hemicellulose is heterogeneous mixture of several types of sugar monomers, both hexoses (*e.g.* mannose, glucose, galactose) and pentoses (*e.g.* xylose and arabinose), and its composition varies in relation to the plant species [21].Cellulose and hemicellulose jointly constitute the holocellulose fraction [22 – 24] of wood. During the early stages of wood decay this carbohydrate fraction is depolymerised mainly by saproxylic fungal activities [25-27], but also from saproxylic insects and soil bacteria community [28]. Specifically, brown-rot fungi are basidiomycetes that have the capacity to depolymerise and metabolise the carbohydrate fraction [29-31] of wood to soluble oligosaccharides or monosaccharides. These fungi remove hemicellulosic xylose and mannose before cellulosic glucose, because cellulose microfibrils are enveloped by hemicellulose [32-33]. However, the composition varies in relation to the type of wood (softwood or hardwood) [34] and the different microorganism activities involved in wood degradation at the various decay stages [35, 36]. Several studies have examined sugar composition in wood [22, 37-40], using various analytical techniques, including the calorimetric method, capillary electrophoresis, gas chromatography (GC) and liquid chromatography (LC) [41, 42]. However, these techniques have their own limitations. GC requires a derivatisation step because of the low sensitivity, low volatility and high polarity and hydrophilicity

of sugar compounds, resulting in longer sample preparation [41, 43]. The LC technique is most widely used for sugar separation, but traditional reverse phase columns do not retain these analytes adequately to perform efficient chromatographic separation and specialised columns are necessary [44]. In the last few years, an analytical technique applied successfully to simultaneous analysis of sugars has been represented by high-performance anion-exchange chromatography, equipped with an integrated pulsed amperometric detector (HPAEC-PAD) [39, 43]. Compared to other LC and GC methods, HPAEC-PAD is a viable way of remedying the main analytical problems related to the specific features of carbohydrates, (*e.g.* their polar and non-volatile nature and the absence of chromophores), without the need for time-consuming preparatory steps such as derivatisation [45]. In consideration of the pKa values of carbohydrates, ranging from 12 to 14, alkaline eluent conditions can transform them into oxyanions, that can be adequately retained on a strongly basic hydroxide form anion exchange column [46, 47] The samples usually have a different sugar concentration, and different dilutions are required to quantify all compounds with PAD analysis. In order to avoid this inconvenience, PAD can be coupled with a charged aerosol detector (Corona CAD), a universal detector able to analyse non-volatile compounds with a response independent of chemical proprieties [48]. To our knowledge, very few papers have described the sugar composition of living wood samples and none that of CWD. In order, to improve knowledge, on decomposition processes in relation to Hunter decay classes [13], the overall aim of this study was to investigate the sugars profile of silver fir CWD samples collected at the different decay stages. Moreover, the variability in sugar content, measured in a single log and in samples from different logs, was evaluated throughout the entire decomposition process. We hypothesized different sugar profiles from the five decomposition classes considered and a gradual decline of cellulose amount with increasing decay stage.

EXPERIMENTAL

Reagents and standards

NaOH 0.1 M was produced by diluting the NaOH 1 M obtained from ampoule concentrates provided by Vetrotecnica (Padua, Italy) 10 times. LC-MS grade methanol, ethanol (≥99.8%) and hydrochloric acid were purchased from Fluka (St. Louis, MO, USA) and chloroform from VWR (West Chester, PA, USA), while toluene and sodium hypochlorite (6%; NaClO) were from Sigma–Aldrich (St. Louis, MO, USA). Sample and eluent solutions were prepared with ultrapure water > 18 Mohm/cm obtained using an Arium®Pro Lab Water System (Sartorius AG, Goettingen, Germany). The standards used for quantitative determination - D-(−)-arabinose (98%), D-(+)-fructose (≥99%), D-(+)-galactose (97%), D-(+)- glucose (99.5%), D-(+)-xylose (99%), sucrose, L-(−)-rhamnose monohydrate (99%), L-(−)-mannose (≥99%), maltose monohydrate (98%), D-(+)-trehalose dihydrate, D-(+)-cellobiose (≥98%), myo-inositol (99%) and D-Sorbitol (98%) - were purchased from Sigma–Aldrich. A stock solution of each sugar was prepared by weighing 10 mg of standard powder into 100 ml of water (100 mg l^{-1}), then mix solutions were obtained for calibration by diluting the stock solution at 0.2, 0.5, 2 and 4 mg L^{-1} and injecting 5 µl of each.

Wood sampling and sample preparation

The study was carried out at two mountain sites in the Italian Alps (Trentino region): Lavarone (45° 56' N and 11° 18' E) and Molveno (46° 09' N and 10° 59' E). Both sites are characterised by silver fir - Norway spruce (*Picea abies* (L.) H. Karst) mixed forest with a prevalence of the former species. Specifically, at both sites and at different seasons, (at the beginning of July 2017 in Lavarone and at the end of October 2017 in Molveno) five living silver fir trees from each area were selected and sampled by taking two radial cores per tree at a height of 1.30 m using a Pressler increment borer. Before chemical analysis, the two radial cores from the same tree were brought together. In addition, in the same areas, two different silver fir CWD samples were obtained. Specifically, initial sampling (at the Lavarone site) was carried out in a circular plot with a 25 m radius, selecting 5 single logs belonging to different decay stages and collecting 5 replicates for each. A second phase of sampling

(at the Molveno site) was performed, in a circular plot with a radius of 55 m, selecting 5 different logs for each decay class (25 in total) and collecting a single replicate for each. In both sampling processes, CWD samples were taken randomly and classified using the five-class decay system of Hunter [13]; (Figure 1). Specifically, CWD samples in decay classes 1, 2 and 3 were taken with a cross-section of about 10 cm diameter using a manual saw; while 20 × 30 cm bags of CWD samples were filled manually for more advanced decay stages (classes 4 and 5). Before chemical analysis, the bark was removed from CWD samples in classes 1 and 2 using a manual saw, and when present, soil particles, litter, moss and fungi attached to the surface of the samples were also removed using a brush. After drying at 80 °C in an oven for 6 h, the samples were cut-milled to 3 mm (IKA mill), transferred into sterile 50 ml falcon tubes (Sartorius AG, Goettingen, Germany) and stored at 4 °C. For the analysis of carbohydrates, 0.2 g of each powdered wood sample were extracted in 4 mL of water-methanol mixture (75:25, v/v). The solutions were first shaken using a Multi Reax (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) for 15 min, and were then placed in an ultrasound bath (Labsonic LBS1–6L, FALC Instruments, Treviglio BG, Italy) for 15 min. After centrifugation (5 min, 4100 rpm), the supernatant was filtered with 0.45 μm PTFE filter cartridges (Sartorius AG) into an analytical 2 mL vial, and 5 μl injected.

Figure 1: CWD samples during the five decay stages according to Hunter (1990).

IC-PAD-CAD analysis

Chromatographic separation was performed using an ICS 5000 ion chromatography system (Dionex -Thermo Scientific, Waltham, USA) equipped with an eluent generator, an autosampler, a quaternary gradient pump, a column oven, PAD consisting of a gold working electrode and a palladium counter electrode and CAD. The separation of carbohydrates (both mono- and di-saccharides) was carried out by injecting 5 μL of sample on a CarboPac PA200 (3 x 250 mm) analytical column, which was preceded by a CarboPac PA200 (3 x 50 mm) guard column (Dionex - Thermo Scientific, Waltham, USA). The column stationary phase was composed of a hydrophobic, polymeric, pellicular resin bounded with quaternary ammonium as anion-exchange functional group. Both columns were operated at a constant temperature of 30 °C. Chromatographic elution was carried out at a flow rate of 0.4 mL/min using an eluent generator that allowed the automatic production of potassium hydroxide (KOH) eluent, controlling the electrical current applied to the electrolysis of deionised water. Isocratic KOH elution at 0.1 mM was run from 0 to 27.5 min, then gradient elution was performed from 0.1 to 100 mM, from 0 to 21.5 min and held until 27.5 min. KOH concentration was then reduced to 0.1 mM, enabling column equilibration for 5 min. Deionised water was constantly purged with helium to avoid the formation of carbonate. Carbohydrate detection was achieved using PAD with the working pulse potential quaternary curve (Table 2), with reference to a saturated Ag/AgCl reference electrode and CAD. For CAD monitoring, a low filter was used and CAD nitrogen gas pressure was adjusted to 35 psi. The eluent, rich in KOH, was not compatible with the CAD detector, and to overcome this problem an anionic electrochemical regenerator suppressor (AERS 500; 2 mm) was used to remove non-volatile solutes.

Table 2: PAD potential and duration with reference to a saturated Ag/AgCl reference electrode.

Time (s)	Voltage (V)	Integration
0	0.95	Off
0.2	0.95	On
0.4	0.95	Off
0.41	-1.15	Off
0.42	-1.15	Off
0.43	1.45	Off
0.44	0.75	Off
0.5	0.75	Off

Method validation

The type and quantity of carbohydrates in CWD samples were analysed and quantified with IC-PAD-CAD. The characteristics of the method were studied using 13 pure standards. For both PAD and CAD detectors, the limit of detection (LOD) was estimated as three standard deviations of ten replicated blank samples according to EURACHEM [49], and similarly the limit of quantification (LOQ) was estimated as ten standard deviations of the same replicates. The linearity and quantitation range of each sugar were evaluated by adopting linear or quadratic regression models between the signal response (peak area) and the nominal concentration of a total of 10 increasing levels from 0.01 to 400 mg L^{-1}, each replicated with 3 different injections. Specifically, 7 levels, from 0.01 to 20 mg L^{-1}, were evaluated for PAD and 5 levels, from 2 to 400 mg L^{-1}, for CAD. The linearity range was defined as the maximum concentration allowing a correlation coefficient (R^2) higher than 0.99, starting from 0.01 mg L^{-1}.

Precision was estimated as the relative standard deviation (RSD%) of 3 analytical replicates of a blank sample spiked at 0.2 mg L^{-1} (PAD) and 100 mg L^{-1} (CAD) of each sugar. Method accuracy, expressed as recovery, was estimated as the percentage difference between the expected and the returned mean concentration of a CWD sample, spiked at 2 mg L^{-1} of each sugar, replicated 4 times. For accuracy, all sugars were evaluated with a PAD detector, with the exception of glucose, which was quantified with CAD.

In order to extend the sugar profiling of CWD samples, unknown sugar screening was performed. Peaks present in PAD chromatograms, but not identified by analytical standards, were reported as 'unknown@RT', with RT corresponding to the peak retention time, and evaluated using the peak area (unit measurement in p-Amperes·minute; pA*min).

α-cellulose determination

α-cellulose determination was performed to verify the decay progression of our wood samples. The analysis was carried out according to the method described by Loader et al. [50] with some modifications. The wood samples were processed using three-step chemical treatments including: 1)

pretreatment through solvent extraction to remove minor components such as resin from wood samples; 2) delignification with a bleaching reaction; 3) purification with a sodium hydroxide solution to eliminate lignin and hemicellulose. In detail, 0.3 g of powdered wood were first weighed and washed three times with 10 ml of toluene/ethanol solution (2:1, v/v) to remove resin. Afterwards, the sample was placed in a thermostatic water bath at 68 °C for 30 min and then centrifuged for 15 min at 4100 rpm. After centrifugation, the supernatant was discarded and the sample was washed with 10 ml of $NaClO_2$ solution for delignification treatment. The sample dispersed in $NaClO_2$ solution was then shaken in a vortex mixer for 1 min and centrifuged again for 30 min at 4100 rpm. After this procedure, the supernatant was discarded and the sample, soaked in 10% NaOH solution, was put into a thermostatic water bath for 30 min at 68 °C. Then, the sample was centrifuged for 15 min at 4100 rpm, and having discarded the supernatant, it was soaked again in 17% NaOH solution for 45 min at room temperature. After that, it was centrifuged for 15 min at 4100 rpm and having discarded the supernatant, was carefully washed twice with 30 ml of distilled water to remove any traces of NaOH. The sample was then dried at 70 °C for 24 h and finally weighed. α-cellulose content was estimated as the difference between the initial sample weight and the final weight after treatment.

Statistical analysis

Statistical analysis of carbohydrates was performed using Statistica 13.1 (Stat- Soft, 2010) and XLSTAT (version 2018.1 Addinsoft, France), establishing all values below the LOD as equal to half the LOD. Data not normally distributed (Kolmogorov–Smirnov test, $p < 0.05$) were normalised by applying Box-Cox transformation. Significant differences between the measured parameters were determined using Tukey's HSD test ($p < 0.05$). Principal component analysis (PCA) was carried out for CWD samples collected at the Lavarone and Molveno sites, to evaluate whether specific sugar profiles could characterise Hunter's classification of CWD during decay stages.

RESULTS AND DISCUSSION

Evaluation of sample extraction

The extraction procedure is a pivotal step for assuring effective identification and quantification of wood constituents, such as carbohydrates. The efficiency of extraction depends on several factors, related especially to the solvents used, extraction time, temperature and composition of the sample [51]. In particular, carbohydrates are highly polar [52] hydrophilic compounds, for this reason polar solvents, such as water, should be preferred for extraction [53-54]. In this work, in order to establish the best carbohydrate extraction conditions, various mixes of methanol/water were used (100% H_2O; H_2O/MeOH at 75:25, 50:50, 25:75; 100% MeOH). Of these, we selected H_2O/MeOH at 75:25, because with a reduced methanol percentage chromatographic separation showed peaks with more symmetrical shapes.

Method validation

The method allowed the quantification of 13 sugars, using linear calibration curves (for myo-inositol, sorbitol, galactose, sucrose, glucose, fructose, mannose, cellobiose and maltose) and quadratic calibration curves (for arabinose, rhamnose, trehalose and xylose) for PAD detection and linear calibration curves for CAD detection that always had correlation coefficients (R^2) higher than 0.99. The range of quantitation went from the quantification limit to 20 mg kg^{-1} for each sugar quantified by PAD and to 400 mg L^{-1} for each sugar quantified by CAD.

The LOD ranged from the lowest values for lactose (0.004 mg kg^{-1}), to the highest for myo-inositol (16.0 mg kg^{-1}). Within-run precision (RSD %) was investigated for each sugar at low (0.2 mg L^{-1} with PAD) and high concentration levels (100 mg L^{-1} with CAD), covering the entire quantification range. Considering the overall group of sugars, the median precision values were 5 and 9 at the corresponding low and high concentrations, respectively, always being lower than 10%, except for sorbitol, mannose and myo-inositol at 0.2 mg L^{-1} (13%, 13% and 15%, respectively) and for sucrose at 100 mg L^{-1} (19%). Accuracy, evaluated in terms of recovery for all sugars, had a median value of 115% and fell within the range of 60-120% for all sugars, except for arabinose, maltose, myo-inositol

and rhamnose. The method characteristics, namely the linearity, LOD, LOQ, precision and accuracy determined for each target sugar compound, are shown in Table 3.

Table 3: Validation parameters for the 16 carbohydrate analytical standards for PAD and CAD detectors.

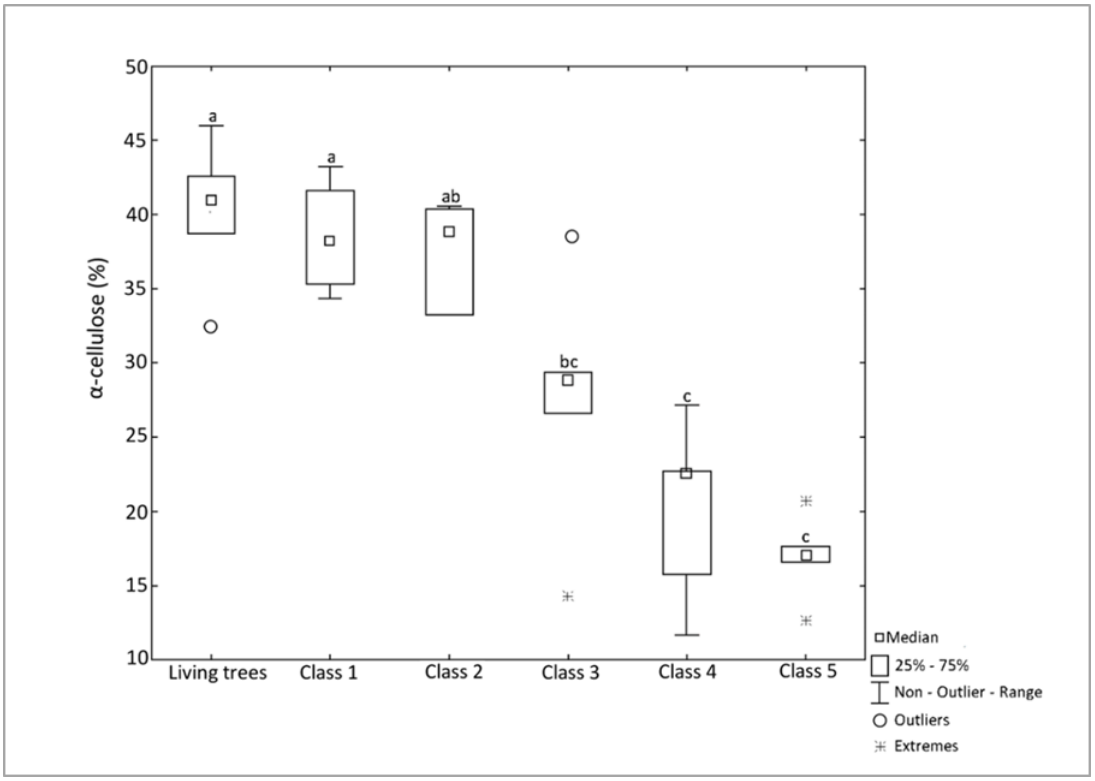
PAD - Compounds	Detector	RT (min)	LOD (mg kg^{-1})	LOQ (mg kg^{-1})	Linearity range (mg kg^{-1})	Precision (RSD %)	Accuracy (%)
Myo-inositol	PAD	2.2	16	53	16 - 20	15	164
Sorbitol	PAD	2.4	0.09	0.32	0.09 - 20	13	63
Trehalose	PAD	2.5	0.03	0.11	0.03 - 20	9	40
Arabinose	PAD	5.1	0.01	0.04	0.01 - 20	3	158
Rhamnose	PAD	5.3	0.03	0.09	0.03 - 20	7	151
Galactose	PAD	5.9	0.04	0.13	0.04 - 20	1	118
Sucrose	PAD	6.1	1.1	3.6	1.1 – 20	1	118
Glucose	CAD	7.2	0.88	5.95	0.88-400	8	99
Xilose	PAD	7.4	0.01	0.03	0.01 – 20	2	113
Mannose	PAD	7.8	0.02	0.08	0.02 - 20	13	116
Fructose	PAD	9.5	0.12	0.41	0.12 – 20	4	72
Cellobiose	PAD	19.9	0.01	0.05	0.01 – 20	5	115
Maltose	PAD	21.7	0.04	0.13	0.04 - 20	5	154

Notes: PAD= pulsed amperometric detector; CAD= charged aerosol detector; LOD= limit of detection; LOQ= limit of quantification; RSD= relative standard deviation.

α cellulose

As expected, the amount of α cellulose gradually decreased with increasing decay (Figure 2). Specifically, comparing the results related to cellulose content in CWD samples, we found a decrease from 44% for CWD in decay class 1 to 19% for CWD in decay class 5. Similar results were found for silver fir and beech in the Apennines [18] and for other coniferous species such as Norway spruce and European Larch (*Larix decidua* Mill.) in an Alpine setting [2, 5]. As CWD decomposition progresses and the content of cellulose decreases, an increase in the content of lignin is expected in later decomposition stages and deadwood is gradually incorporated in the organic soil layers. Fungal communities undergo a parallel succession of taxa; in the first phases of CWD decay processes, white rot fungi predominate, for being replaced by brown rot fungi (able to use cellulose) and later by an increasing fraction of Basidiomycota (able to use lignin) [55, 56]. In particular, for the gymnosperm wood after a first phase dominated by bacteria and molds, the decay process is preferentially driven by brown rot fungi able to degrade the polysaccharides and leave the lignin fraction only partially modified [56]. Analytical methods of separating and identifying decomposition phases may implement common visual classification, when more accurate detection of habitat changes and estimate for carbon sink are required in forest ecosystem analysis.

Figure 2: Box plots with amounts of α cellulose for living trees and CWD in decay classes. Different letters indicate significant differences between decay classes using Tukey's HSD test ($p < 0.05$).



Carbohydrates in wood cores of living silver fir trees

Several carbohydrates were found in wood cores of five living silver fir trees (Lavarone site; July 2017). As reported in Table 4, six monosaccharides (arabinose, fructose, galactose, glucose, mannose and xylose), four disaccharides (cellobiose, maltose, sucrose and trehalose) and two sugar alcohols (myo-inositol and sorbitol) were found in detectable amounts. Rhamnose was always under the detection limit. Specifically, in monosaccharides, fructose and glucose were the main sugars detected in wood cores, with a concentration ranging from 126 to 220 mg kg^{-1} and from 189 to 319 mg kg^{-1} respectively, while in disaccharides the main sugar was sucrose, with a concentration ranging from 211 to 804 mg kg^{-1}. To the best of our knowledge, there are few studies covering carbohydrate (*e.g.* monosaccharides and disaccharides) composition in silver fir wood; however, in accordance with our results, Saranpää et al. [57] reported sucrose, glucose and fructose as the main carbohydrates found in stem wood of some coniferous species such as *Pinus sylvestris* L. As expected, the concentration of galactose and mannose (from 11 to 17 mg kg^{-1} and from LOD to 9 mg kg^{-1}, respectively) was found to be slightly higher than that of arabinose and xylose (from 2 to 5 mg kg^{-1} and from the LOD to 1.7 mg kg^{-1}, respectively), considering that galactoglucomannan is the predominant hemicellulose type in softwood species, including the silver fir [58,59]. These differences were also observed by Bertaud and Holmbom [60] in a study concerning the carbohydrate composition of Norway spruce stem sections. Moreover, Álvarez et al. [61] showed that pentoses, such as arabinose and xylose, are more abundant in hardwood than softwood, due to higher xylose content in hardwood.

In addition, cellobiose (from 0.9 to 21 mg kg^{-1}), maltose (from 20 to 27 mg kg^{-1}) and trehalose (5.2 to 14 mg kg^{-1}) have also been found. Specifically, Chen [24] showed that together with glucose, cellobiose represents the basic unit in the cellulose polymer in woody plants, while Dhuli et al. [62] showed the presence of maltose in buds and needles of silver fir. As regards trehalose, some studies have reported the occurrence of this sugar in vascular plants [63-65], whereas other studies have reported the presence of trehalose in the wood of some coniferous species (e.g. Norway spruce and Scots pine), correlated with fungal infections [66-68]. Of sugar alcohols, myo-inositol was the most abundant, with

concentrations ranging from 40 to 80 mg kg^{-1}. Dhuli et al. [62] reported the presence of myo-inositol in the buds and needles of various conifers, including silver fir.

As regards the wood cores collected from silver fir trees in Molveno (October 2017), a similar sugar profile to that observed in wood samples collected in Lavarone in July was found. Four disaccharides (sucrose, maltose, trehalose and cellobiose), six monosaccharides (glucose, fructose, galactose, arabinose, mannose and xylose) and two sugar alcohols (myo-inositol and sorbitol) were detected, albeit at different concentrations (Table 4).

Rhamnose was still under the LOD. As for the Lavarone site, of the monosaccharides, fructose (from 127 to 575 mg kg^{-1}) and glucose (from 385 to 3036 mg kg^{-1}) were confirmed to be the main sugars, with concentrations significantly higher in wood samples collected in October than those collected in July. The galactose and mannose concentration (from 4 to 47 mg kg^{-1} and from 1 to 5 mg kg^{-1}, respectively) was slightly higher than that of arabinose and xylose (from 1 to 4 mg kg^{-1} and from 0.02 to 2 mg kg^{-1}, respectively). However, on comparing the two sites, the galactose and mannose concentration was not significantly higher in wood samples collected in October than in those collected in July. The arabinose concentration was significantly lower in wood samples collected in October, while the xylose concentration was significantly higher in wood samples collected in October than in those collected in July. As regards disaccharides, sucrose was confirmed to be the most common, with a concentration ranging from 290 to 1955 mg kg^{-1}. On comparing the two sites, sucrose was slightly but not significantly higher in wood samples collected in October than those collected in July, essentially confirming previous observations on seasonal changes in carbohydrate content in woody species, with the lowest amounts during the growing season and the highest late in the season or during dormancy [69,70]. Cellobiose, maltose and trehalose, with concentrations ranging from the LOD to 5 mg kg^{-1}, from the LOD to 54 mg kg^{-1} and from 6 to 386 mg kg^{-1}, respectively, did not show any significant differences at the two sites.

Of the sugar alcohols, myo-inositol content, despite reaching the highest concentration (from the LOD to 39 mg kg^{-1}), was significantly lower in Molveno samples in July than in the wood cores collected

in Lavarone. Conversely, sorbitol, which had concentrations ranging from 0.02 to 23 mg kg^{-1}, was slightly but not significantly richer in the Molveno samples. In accordance with previous studies, our results confirm that changes in carbohydrate content can be associated with seasonal variations in acclimation and deacclimation in woody species [71,72]. In addition to the above-mentioned carbohydrates, we also detected two unknown compounds on PAD chromatograms, reported as unknown@15.9 and unknown@19.88, as shown in Table 4. Specifically, in wood samples collected in Lavarone in July, we found only unknown@19.88, with an area signal (pA*min) ranging from 0.01 to 0.04, while in wood samples collected in Molveno in October, we also found unknown@15.9, with an area signal (pA*min) ranging from 0.4 to 2. The signal of unknown@19.88 did not show any significant differences between the two sites.

Table 4: Statistical distribution of carbohydrates detected in wood samples collected at the Lavarone and Molveno sites from living silver fir trees. Data are expressed in mg kg-1 for carbohydrate compounds and in pA*min for unknown compounds.

Compounds	Living trees (N=5) Lavarone site			Living trees (N=5) Molveno site		
	Min	**Mdn**	**Max**	**Min**	**Mdn**	**Max**
Monosaccharides						
Arabinose	2	4[a]	5	1	1[b]	4
Fructose	126	137[b]	220	127	372[a]	575
Galactose	11	13[a]	17	4	31[a]	47
Glucose	189	261[b]	319	385	1739[a]	3036
Mannose	<LOD	<LOD[a]	9	1	5[a]	9
Rhamnose	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Xilose	<LOD	<LOD[a]	1.7	0.02	0.02[b]	2
Disaccharides						
Cellobiose	0.9	10[a]	21	<LOD	1[a]	5
Maltose	20	25[a]	27	<LOD	31[a]	54
Sucrose	211	427[a]	804	290	1409[a]	1955
Trehalose	5	7[a]	14	6	8[a]	386
Sugar alcohols						
Myo-inositol	40	51[a]	87	<LOD	<LOD[b]	39
Sorbitol	<LOD	<LOD[a]	8	<LOD	6[a]	23
Unknown						
Unknown@15.9	n.d.	n.d.	n.d.	0.4	1	2
Unknown@19.88	0.01	0.02[a]	0.04	n.d.	n.d[a].	0.1

Notes: LOD = limit of detection; Min = minimum content; Mdn = median content; Max = maximum content; n.d.= not detected.

Carbohydrates in coarse woody debris

In this part of study, we investigated for the first time to the best of our knowledge, changes in the sugar profiles of silver fir coarse woody debris in relation to Hunter's decay stages. Specifically, we first studied sugar content variability in wood samples collected from a restricted plot (Lavarone) from a single log, considering logs at the 5 different stages. We then investigated sugar content variability in wood samples collected from a wider plot (Molveno) from different logs, considering logs at the 5 different stages. The sugar content found in CWD samples collected in Lavarone and Molveno are shown in Table 5, while the trend and variability in sugar content in the different decay classes can be observed in Figure 3. In the Lavarone samples, sucrose and rhamnose were always under the LOD in all the samples analysed. As regards monosaccharides, arabinose had an interesting accumulation at the 2nd decay stage, with content ranging from 135 to 169 mg kg^{-1}, and a decrease in further decay classes, while mannose showed an evident trend, with a steady decrease after the 1st stage (from 137 to 262 mg kg^{-1}) during the decay process.

As regards disaccharides, cellobiose showed a decreasing trend, with concentrations significantly higher in the 1st (from 9 to 36 mg kg^{-1}) and 2nd decay classes (from 6 to 30 mg kg^{-1}) compared to later decay stages. This sugar derives from the cellulose degradation induced by enzymes secreted by several wood-rotting basidiomycetes fungi [73], and in our study we indeed confirmed progressive cellulose degradation over the decay stages [2,5]. Maltose showed an accumulation trend during decay, reaching significantly higher content in the 4th class (from 40 to 70 mg kg^{-1}) and 5th class (from 6 to 94 mg kg^{-1}) than in earlier stages, whereas trehalose decreased slightly during the decay process. The presence of trehalose is related to the metabolism of many organisms, such as bacteria and fungi, responsible for the decomposition of deadwood [66, 74-76].

Of the sugar alcohols, myo-inositol always had very low content and there were no significant differences between CWD decay classes, while sorbitol showed a trend for accumulation during wood degradation, with the highest content in the 4th decay class (from 7 to 23 mg kg^{-1}) but decreasing content in the 5th.

In addition to the unknown compounds detected in wood cores of silver fir trees (unknown@15.9 and unknown@19.88), we also identified a 3rd unknown compound, unknown@21.64. These compounds showed a similar decreasing trend during different decay stages. In particular, unknown@15.9 was significantly higher in the 1st decay class, unknown@19.88 was significantly higher in the 1st and 2nd decay classes and unknown@21.64 was significantly higher in the 1st, 2nd and 3rd decay classes compared with the other decay classes.

As regards CWD samples collected at the Molveno site from different logs, a similar sugar profile to that observed in the CWD samples collected at the Lavarone site was found in different decay classes. Six monosaccharides (arabinose, fructose, galactose, galactose, mannose and xylose), four disaccharides (cellobiose, maltose, sucrose and trehalose) and two sugar alcohols (myo-inositol and sorbitol) were detected, albeit at different concentrations, while as for the Lavarone site, sucrose and rhamnose were still under the LOD in all the samples analysed. Surprisingly, at this site, the concentration of fructose was also under the LOD in all samples. Previous studies have shown that fructose represents a possible carbon source for the growth of decomposer organisms such as fungi [77,78]. The low concentration of fructose detected in our study can probably be associated with a higher metabolic consumption of this sugar by fungi. As regards monosaccharides, arabinose, galactose, glucose xylose and mannose were quantified, but without any significant differences between CWD decay classes. However, on comparing the results between the two sites, we observed that arabinose, despite showing a higher concentration in the Molveno samples (from 118 to 855 mg kg^{-1}), had a similar trend during decay stages.

Of the disaccharides, cellobiose showed a decreasing trend with high content in the 1st decay class (0.3 to 6 mg kg^{-1}) compared to the later decay stage.

A similar sugar trend was observed previously for the Lavarone site. Maltose had high content in later decay stages (up to 32 mg kg^{-1}), however without any significant differences between CWD decay classes. A similar sugar trend was observed previously for the Lavarone site, although with concentrations up to three times higher than for Molveno CWD. Trehalose, the disaccharide present

at the highest concentration, showed a decreasing trend, with content significantly higher in the 1st (from 197 to 1678 mg kg^{-1}) and 2nd decay classes (from 352 to 1091 mg kg^{-1}) compared to later decay stages. Similar trends were observed previously for the Lavarone site, albeit with concentrations five times lower. These results can be explained by the action of decomposer organisms, such as fungi, during decomposition processes [67]. In the early phases of wood decomposition, a rapid loss of easily soluble sugar (and other compounds, including starch and amino acids) occurs due to leaching process and microorganism activity, which is followed by a second phase in which lignin and cellulose become the main compounds and are slowly degraded by specialized decomposers [55].

As regards sugar alcohols, we observed that myo–inositol always had much higher content (from 118 to 1391 mg kg^{-1}) than sorbitol (from the LOD to 40 mg kg^{-1}), but the concentrations of these sugars were not significantly different in CWD decay classes. As for the Lavarone site, the unknown compounds, unknown@15.9, unknown@19.88 and unknown@21.64, showed a similar decreasing trend during different decay stages. In these compounds, only the signal of unknown@19.88 showed significant differences in decay classes. The different sugar content in the two sites detected in our study can be explained by the different environmental conditions, such as temperature and moisture, influencing the composition and the activity of decomposer organisms such as fungi [79-81]. Fukasawa et al. [82] showed that the composition of holocellulose can vary during wood decay stages in relation to the role of fungal decomposition of woody debris in forests; holocellulose is selectively decomposed in advanced stages and lignin compounds accumulate. Because of these changes in chemical conditions, deadwood decay rates may vary from fast in the beginning to progressively slower rates, as decomposition proceeds, reflecting the succession of fungal communities with distinct decaying capacity [55]. The variability of each sugar content detected during Hunter decay stages, was evaluated as percentage of relative standard deviation (RSD %) calculated for each stage in both sites. The RSD% values obtained ranged on average from 13% to 189 % for the sugars detected in samples from a same log in Lavarone site, while for the sugars detected from different logs (Molveno) the RSD% values ranged on average from 47% to 168 %. Sugar variability of samples

from a same log was generally found significantly lower than that of samples from different logs (Kolmogorov–Smirnov test, $p < 0.05$): arabinose (13 *vs* 47%), glucose (47 *vs* 115%), maltose (53 vs 168%) and cellobiose (60 *vs* 104 %). Nevertheless myo- inositol (189 vs 66%) showed an opposite behavior.

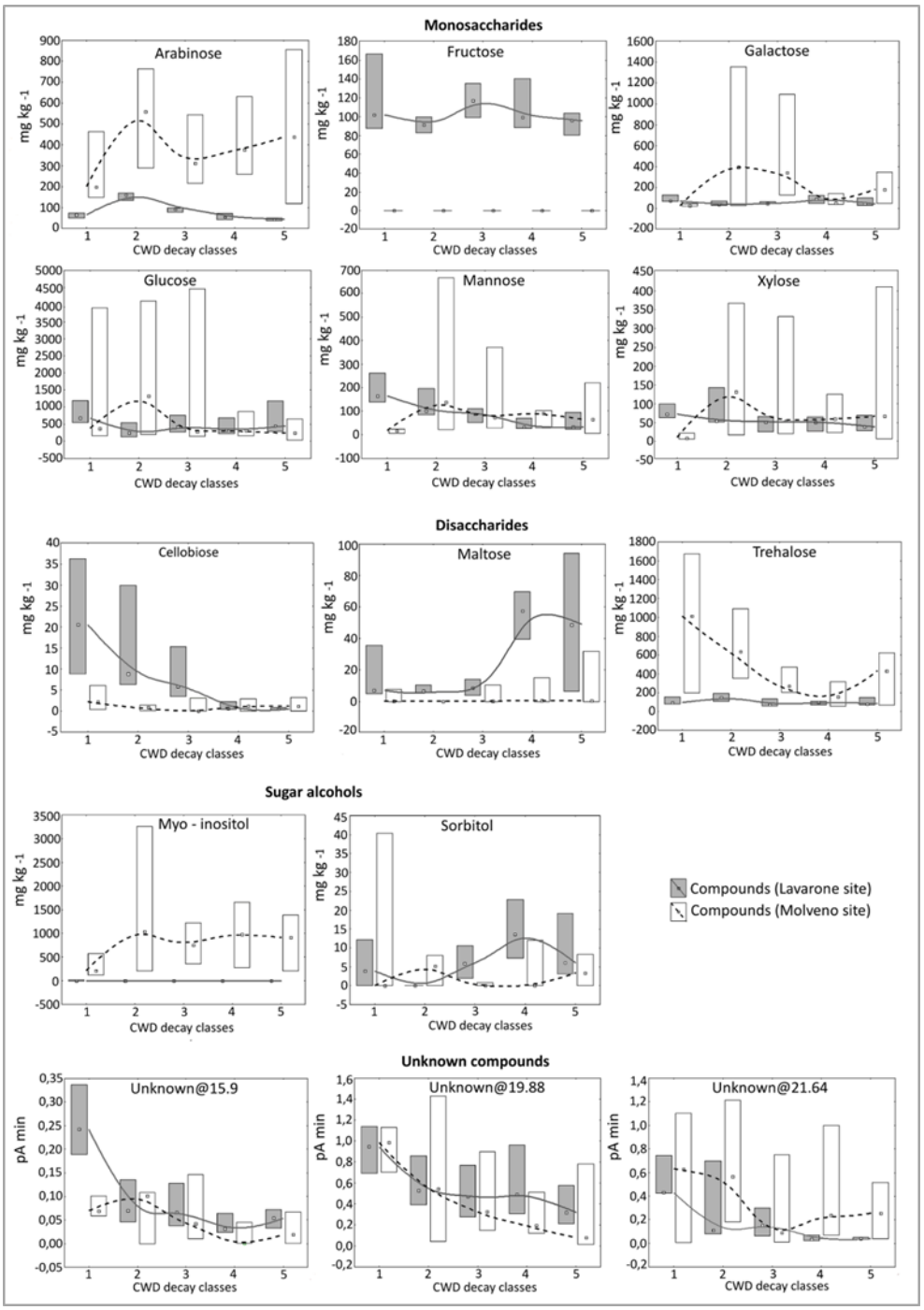
Table 5: Statistical distribution of carbohydrates detected in CWD samples collected at the Lavarone site from a single log and at the Molveno site from different logs. Data are expressed in mg kg-1 for carbohydrate compounds and in pA*min for unknown compounds.

Compounds (Lavarone site)	Class 1 (N=5)			Class 2 (N=5)			Class 3 (N=5)			Class 4 (N=5)			Class 5 (N=5)		
	Min	**Mdn**	**Max**	**Min**	**Mdn**	**Max**	**Min**	**Mdn**	**Max**	**Min**	**Mdn**	**Max**	**Min**	**Mdn**	**Max**
Monosaccharides															
Arabinose	48	65^{c}	71	135	160^{a}	169	76	91^{b}	99	43	54^{c}	72	38	45^{c}	50
Fructose	87	102^{a}	166	83	91^{a}	99	99	117^{a}	135	89	99^{a}	140	80	96^{a}	103
Galactose	71	73^{a}	132	33	40^{a}	72	46	50^{a}	67	49	88^{a}	126	34	45^{a}	104
Glucose	555	674^{a}	1171	127	243^{a}	533	275	422^{a}	747	222	336^{a}	687	278	440^{a}	1180
Mannose	137	165^{a}	262	84	100^{ab}	195	51	88^{bc}	111	26	33^{c}	70	24	33^{c}	94
Rhamnose	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Xilose	63	73^{a}	100	52	55^{a}	143	27	52^{a}	66	28	51^{a}	65	29	39^{a}	68
Disaccharides															
Cellobiose	9	21^{a}	36	6	9^{ab}	30	3	6^{bc}	15	0.5	1^{bc}	2	0.4	0.5^{c}	1
Maltose	5	7^{b}	35	5	7^{b}	10	4	8^{b}	14	40	58^{a}	70	6	49^{a}	94
Sucrose	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Trehalose	81	96^{ab}	157	111	145^{a}	193	59	77^{b}	138	72	101^{ab}	109	72	86^{ab}	151
Sugar alcohols															
Myo-inositol	<LOD	<LOD^{a}	12	<LOD	<LOD^{a}	4	<LOD	<LOD^{a}	1	<LOD	<LOD^{a}	0.4	<LOD	<LOD^{a}	11
Sorbitol	<LOD	4^{bc}	12	<LOD	<LOD^{c}	0.2	2	6^{abc}	11	7	14^{a}	23	3	6^{ab}	19
Unknown															
Unknown@15.9	0.19	0.24^{a}	0.34	n.d.	0.1^{b}	0.1	0.04	0.07^{b}	0.1	0.02	0.03^{b}	0.06	0.03	0.05^{b}	0.07
Unknown@19.88	1	1^{a}	1	0.4	1^{ab}	1	0.3	0.5^{b}	0.8	0.3	0.5^{b}	1	0.2	0.3^{b}	0.6
Unknown@21.64	0.4	0.4^{a}	1	0.1	0.1^{a}	1	0.06	0.15^{a}	0.30	0.02	0.04^{b}	0.07	0.03	0.04^{b}	0.05

Compounds (Molveno site)	Class 1 (N=5)			Class 2 (N=5)			Class 3 (N=5)			Class 4 (N=5)			Class 5 (N=5)		
	Min	Mdn	Max	Min	Mdn	Max	Min	Mdn	Max	Min	Mdn	Max	Min	Mdn	Max
Monosaccharides															
Arabinose	149	197[a]	462	288	559[a]	763	216	312[a]	543	259	377[a]	631	118	438[a]	855
Fructose	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Galactose	22	27[a]	46	30	394[a]	1360	129	344[a]	1093	43	58[a]	144	49	183[a]	350
Glucose	153	365[a]	3902	199	1317[a]	4109	136	280[a]	4467	152	315[a]	871	30	241[a]	657
Mannose	6	17[a]	23	23	137[a]	667	29	73[a]	372	28	91[a]	104	6	65[a]	221
Rhamnose	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Xilose	7	10[a]	22	17	132[a]	368	21	54[a]	332	24	59[a]	125	7	67[a]	411
Disaccharides															
Cellobiose	0.3	2[a]	6	0.1	1[a]	1	<LOD	0.02[a]	3	<LOD	1[a]	3	<LOD	1[a]	3
Maltose	<LOD	0.2[a]	8	<LOD	<LOD[a]	0.3	<LOD	0.2[a]	10	<LOD	0.5[a]	15	<LOD	0.5[a]	32
Sucrose	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Trehalose	197	1014[a]	1678	352	640[ab]	1091	203	268[b]	470	61	159[b]	319	68	431[b]	625
Sugar alcohols															
Myo-inositol	118	213[a]	574	201	1038[a]	3267	363	755[a]	1224	279	979[a]	1659	204	916[a]	1391
Sorbitol	<LOD	<LOD[a]	40	<LOD	5[a]	8	<LOD	<LOD[a]	1	<LOD	<LOD[a]	12	<LOD	3[a]	8
Unknown															
Unknown@ 15.9	0.1	0.1[a]	0.1	n.d.	0.1[a]	0.1	n.d.	n.d.	0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Unknown@ 19.88	0.7	1[a]	1	0.04	0.5[ab]	1	0.1	0.3[ab]	0.9	0.1	0.2[ab]	0.2	n.d.	n.d.	0.8
Unknown@21.64	0.01	0.6[a]	1	0.2	0.6[a]	1	n.d.	0.1[a]	0.8	0.1	0.2[a]	1	n.d.	0.3[a]	0.5

Notes: LOD = limit of detection; Min = minimum content; Mdn = median content; Max = maximum content; n.d.= not detected.

Figure 3: Box plots with carbohydrate concentrations (mg kg^{-1}) and unknown compounds (pA*min) in CWD samples collected at the Lavarone site from the same log and at the Molveno site from different logs. The grey and black lines highlight sugar trends during different decay stages.



Principal component analysis (PCA)

PCA was applied separately to the sugar content of CWD samples collected in Lavarone and in Molveno, in order to assess whether sugar profiles could characterise Hunter's five decay stages. For the Lavarone samples, obtained from a same log, PCA revealed good differentiation between the five different decay classes (Figure 4), and the variance explained by factor 1 and 2 accounted for 82%. CWD samples belonging to the 1st, 2nd and 3rd decay classes were well clustered, while partial overlapping was observed for CWD belonging to the 4th and 5th decay classes. This overlap, corresponding to CWD samples quite similar compositionally, could be explained by a final state of chemical degradation of the wood and corresponding relatively more stable sugar profiles and recalcitrant complexes [83]. Specifically, high content of cellobiose and unknown@15.9 characterised the 1st decay class, a high concentration of arabinose characterised the 2nd decay class, while high concentrations of maltose and sorbitol characterised the 4th and 5th decay classes.

In Molveno samples, the variability of CWD belonging to the same stage was definitely much higher, and PCA explained only 53.5% of total variability, with 1st and 2nd decay stage samples tending to differentiate compared to subsequent decay classes (Figure 5). In particular, these stages were characterised by a high content of trehalose, cellobiose and unknown@15.9.

Figure 4: Principal Component Analysis with the distribution of CWD samples collected at the Lavarone site according to Hunter's decay classes based on the carbohydrate profile.

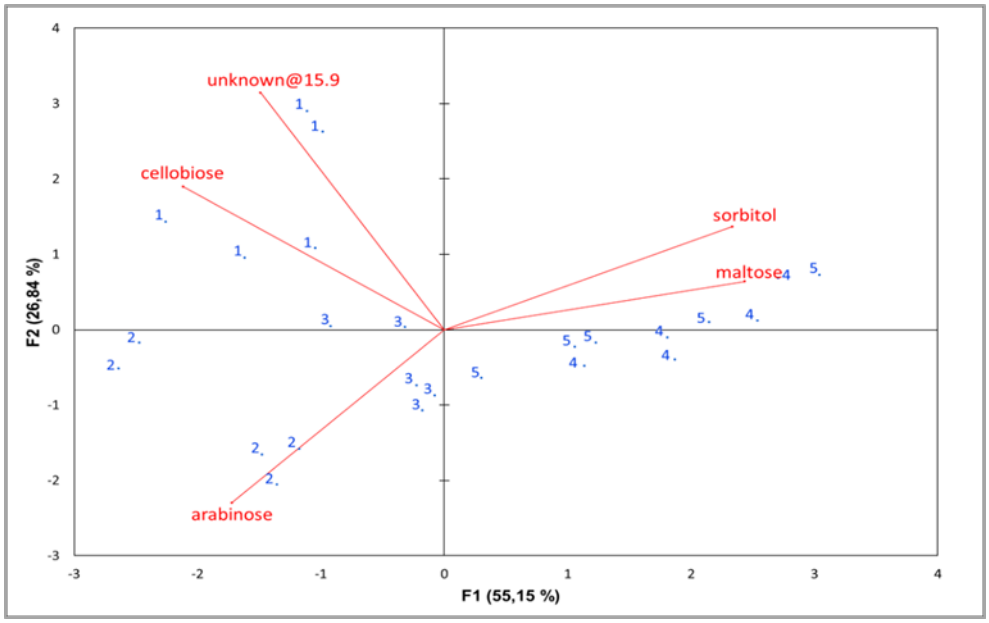
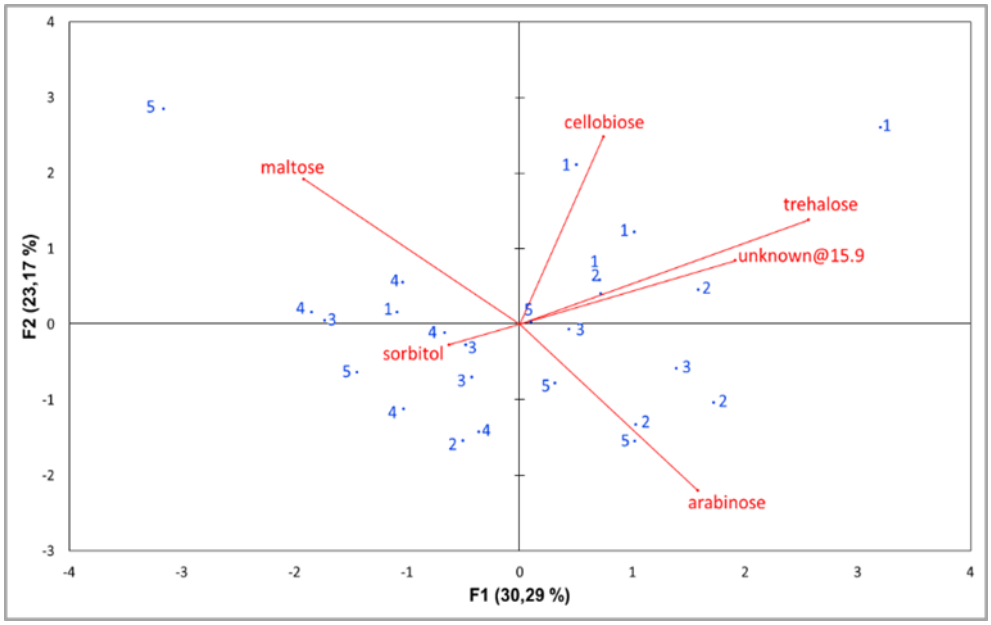


Figure 5: Principal Component Analysis with the distribution of CWD samples collected at the Molveno site according to Hunter's decay classes based on the carbohydrate profile.



CONCLUSION

In conclusion, the IC-PAD-CAD technique proved to be useful for broad identification and sensitive quantification of carbohydrates present both in living wood from silver fir trees and in CWD belonging to all five of Hunter's decay classes. This study also made it possible to establish the variability and trends for sugar content in CWD sampled at two different forest sites and two different times. Ultimately, chemical diversity in decomposing CWD implies biological diversity in forest soils, because of changing sugar patterns driving organism users, which warrants investigations on the effects of forestry practices on topsoil biodiversity and related indicators. Further studies on the specific impact of natural decomposer organisms, such as bacteria, saproxylic insect and fungal communities [84,85], which gain metabolic energy from sugars, would be useful to better understand the specific trend for accumulation and consumption of the studied sugars during the wood debris decomposition process.

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CAPITOLO 3

Fungal impact on lignin degradation and simple phenols formation in silver fir decaying wood

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Abstract

White rot fungi degrade and mineralize lignin through secretion of strong oxidative ligninolytic enzymes, preventing accumulation of dead plant organic matter. Due to the specific structures, different lignolytic enzymes has different mechanism in lignin degradation/oxidation, producing many phenolic compounds. This work explores the phenolic compounds produced at different times by nine fungal species, belonging to the *Heterobasidion* and *Armillaria* genera, before and after their inoculation on silver fir (*Abies alba* Mill.) sawdust under controlled conditions. A total of 81 samples (three replicates for each fungal species and other three replicates kept as a control) were analysed using high-performance liquid chromatography coupled to a hybrid quadrupole-orbitrap mass spectrometer. Eighteen phenolic compounds, including simple phenols, alkylphenyl alcohols, hydroxybenzoketones, hydroxycinnamaldehydes, hydroxybenzaldehydes, hydroxyphenylacetic acids, hydroxycinnamic acids, hydroxybenzoic acids and hydroxycoumarins were detected. In particular, coniferyl alcohol, ferulic acid, p-coumaric acid, acetovanillone, vanillic acid, etc. showed a decreasing trend during degradation process, by contrast an accumulation trend was observed for protocatechuic acid, syringic acid and scopoletin. These results suggested different strategies of silver fir lignin degradation by selected fungal species. PCA revealed a good differentiation between

phenolic compounds and the activity of nine fungal species during the three times of silver fir sawdust degradation.

Keywords: *Abies alba* Mill, *Armillaria* and *Heterobasidion* genera, lignin decay, phenolic compounds, white rot fungi.

Introduction

Lignin constitutes the second largest sink of fixed carbon after cellulose, and its biodegradation is essential for quantifying and monitoring the carbon retention in forest ecosystems (Eggert et al. 1996). Lignin is a complex phenolic biopolymer that plays a central role in mechanical support of plant cell wall, in water transport and pathogen resistance in plants (Liu et al. 2018). It is closely associated with cellulose and hemicellulose components in the plant cell walls, and comprises 10–35% of the dry weight of lignocellulose (Bugg et al. 2011). The lignin molecule is composed of three different phenylpropane monomer units (monolignols), namely *para*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol linked by ether and carbon-carbon type bonds (Watkins et al. 2015). The composition and amount of this polymer varies in relation to the different tree species and even in the different tissues of the same tree (Campbell et al. 1996). Conifers are known to contain high amounts of lignin consisting mainly of guaiacyl units (90%) derived from coniferyl alcohol; hardwoods and herbaceous species contain similar amounts of both guaiacyl and syringyl units derived from sinapyl alcohol (Mäkelä et al. 2015). Moreover, softwood lignin is estimated to comprise about from 19 to 26% of phenolic units, whereas hardwood lignin contains about from 14 to 18% of phenolic units (Brunow and Lundquist 2010). Due to the chemically complex structure, lignin polymer is highly resistant to physical, chemical and biological degradation (Sanchez 2009). White rot fungi are considered the only organisms, which are able to *completely decompose* lignin (Pioli et al. 2018) to CO_2 and water (Kubartova et al. 2015; Sahadevan et al. 2016). In order to depolymerize and mineralize the complex lignin molecule, these fungi secrete various combinations of strong oxidative lignolytic enzymes known as “ligninases” (Arnstadt et al. 2016). Ligninases include mainly lignin peroxidase, manganese

dependent peroxidase and laccase (Wong et al. 2009; Dashtban et al. 2010). In particular, lignin peroxidase directly attack the non-phenolic lignin units, such as veratryl alcohol (VA), by producing intermediate radicals (Martìnez 2002; Hammel and Cullen 2008), whereas manganese-dependent peroxidase and laccase are able to oxidize the phenolic lignin units to phenoxy radicals, leading to the decomposition of the woody structures (Gold et al. 2000; Munk et al. 2015). Different combinations of these enzymes produced by white rot fungi underlie different mechanisms of lignin degradation with the production of various phenolic compounds (Camarero et al. 1994; Sahadevan et al. 2013; Adeboye et al. 2014). For example, the degradation of guaiacyl-β-coniferyl ether in softwood lignin (e.g. pine and spruce lignins) leads to the formation of coniferyl alcohol, coniferylaldehyde, ferulic acid, several low molecular weight aromatic acids and aldehydes including vanillin and vanillic acid (Nord 1964; Kuiters 1990; Hofrichter 2002; Azarpira et al. 2014). Other common degradation products are syringic acid, syringaldehyde, protocatechuic acid and gallic acid. Various chromatographic techniques were used to identify and quantify the phenolic compounds of lignin, including gas chromatography (GC), liquid chromatography (LC), size exclusion chromatography (SEC), capillary electrophoresis (CE) and two-dimensional (2D) chromatography (Abdelaziz et al. 2016). However, among these analytical techniques, the coupling of high-performance liquid chromatography and mass spectrometry (HPLC-MS) proved to be a powerful technique for the analysis of low molecular weight compounds in lignin samples with high selectivity and sensitivity (Abdelaziz et al. 2016).

Many studies have extensively investigated the role and activity of ligninolytic enzymes produced by white rot fungi, such as *Phanerochaete chrysosporium* during lignin depolymerization (Ulmer et al. 1983; Cai et al. 1993; Wyatt et al. 1995; Zeng et al. 2014). Several studies have also described different strategies of fungi in metabolizing the phenolic compounds during lignin degradation in plants (Ander et al. 1980; Eriksson et al. 1990; Mäkelä et al. 2014); however, none of them has focused on silver fir wood (*Abies alba* Mill.), one of the most valuable conifer species in Europe. Silver fir is widely distributed in Central European forests and it is consequently of significant

ecological and economic value (Dobrowolska et al. 2017). This work aimed to study how the different enzymatic pools of the various fungi influenced the degradation of the lignin of this type of wood and, therefore, its wood rate decomposition. The study was conducted in the laboratory under controlled condition, collecting the samples at different times during six months. Nine different species of white rot fungi, belonging to the *Heterobasidion* and *Armillaria* genera specialised in decaying coniferous wood (Capretti et al. 1990; Oliva et al. 2009), were inoculated on silver fir sawdust samples. Phenolic profiles, before and after the inoculation, were explored using a high-performance liquid chromatography coupled to a hybrid quadrupole-orbitrap mass spectrometer (LC-Q-Orbitrap).

Materials and methods

Reagents and standards

LC-MS grade acetonitrile (99.9%), LC-MS grade methanol (99.9%), and MS grade formic acid (98%) were purchased from Fluka (St. Louis, MO, USA). Potato dextrose agar (PDA) was purchased from Oxoid (Hampshire, England). Deionized water used for the preparation of the sample and eluent solutions was obtained using an Arium®Pro Lab Water System (Sartorius AG, Goettingen, Germany). The phenols standards used for quantitative determination were grouped into nine classes according to their chemical structure (Neveu et al. 2010; Table 1). Eight water-methanol stock solutions of almost 5-6 phenols, each of 100 mg l^{-1} (Table 1), were prepared. Methanol content ranged from 15 to 55% according to the component solubility. The stock solutions were then combined in a single intermediate solution (water-methanol mixture; 75:25 v/v), with a concentration of 10 mg l^{-1} for each phenol and fresh diluted, to the desired concentration, before each analysis. Stock solutions were stored at – 4 °C. Mass calibration solution (Pierce® ESI Negative Ion Calibration Solution) was purchased from Thermo Fischer Scientific Inc. (Waltham, MA, USA).

Table 1: Technical characteristics of phenolic analytical standards.

Phenolic compounds			Stock solution	
	Purity	Supplier	I.D.	MeOH%
Simple phenol				
phenol	≥ 99%	Sigma Aldrich	4	22
pyrocatechol	≥ 99%	Sigma Aldrich	3	22
Alkyphenols				
4-ethylcatechol	≥ 98%	Sigma Aldrich	4	55
4- methylcatechol	≥ 98%	Fluka	5	22
4- vinylphenol	n.d.	Sigma Aldrich	5	55
m-cresol	≥ 98%	Sigma Aldrich	5	55
o-cresol	≥ 99%	Sigma Aldrich	5	35
p-cresol	≥ 99.9%	Sigma Aldrich	6	55
Methoxy and alkylmethoxyphenols				
4-ethylguaiacol	≥ 98%	Sigma Aldrich	6	55
4-methylguaiacol	≥ 99%	Sigma Aldrich	6	55
4- vinylguaiacol	≥ 98%	Sigma Aldrich	6	55
guaiacol	≥ 99%	Sigma Aldrich	6	55
Dimethoxyphenol and alkylphenylmethoxy acohols				
4-methylsyringol	≥ 97%	Sigma Aldrich	7	55
syringol	≥ 99%	Sigma Aldrich	7	35
Alkyphenyl alcohols				
coniferyl alcohol	≥ 98%	Sigma Aldrich	3	22
hydroxytyrosol	≥ 98%	Sigma Aldrich	7	22
homovanillyl alcohol	≥ 99%	Sigma Aldrich	5	35
Hydroxyphemylpropenes				
eugenol	≥ 99%	Fluka	7	55
isoeugenol	≥ 98%	Sigma Aldrich	8	35
Hydroxybenzoketones				
acetosyringone	≥ 97%	Sigma Aldrich	1	40
acetovanillone	≥ 98%	Sigma Aldrich	3	40
ethyl vanillate	n.d.	Sigma Aldrich	8	35
isoacetosyringone	≥ 97%	Sigma Aldrich	3	40
isoacetovanillone	≥ 97%	Sigma Aldrich	8	40
isopropiovanillone	≥ 96%	Sigma Aldrich	7	40
methyl vanillate	≥ 99%	Sigma Aldrich	2	40
Hydroxybenzoether				
vanillyl ethyl ether	n.d.	Fluka	8	35
Hydroxycinnamaldehydes				
coniferylaldehyde	≥ 98%	Sigma Aldrich	2	55
sinapinaldehyde	≥ 98%	Sigma Aldrich	4	55
Hydroxybenzaldehydes				
syringaldehyde	≥ 98%	Sigma Aldrich	2	40
vanillin	≥ 99%	Sigma Aldrich	1	40
Hydroxyphenylacetic acids				
homovanillic acid	≥ 98%	Sigma Aldrich	1	15
Hydroxycinnamic acids				
caffeic acid	≥ 95%	Fluka	4	15
ferulic acid	≥ 98%	Fluka	2	15
p-coumaric acid	≥ 98%	Sigma Aldrich	1	15
Hydroxybenzoic acids				
benzoic acid	≥99.5%	Sigma Aldrich	3	15
gallic acid	≥97.5%	Sigma Aldrich	4	15
gentisic acid	≥ 98%	Fluka	4	15
p-carboxyphenol acid	≥ 99%	Fluka	1	15
protocatechuic acid	≥ 97%	Fluka	2	15

syringic acid	≥ 97%	Sigma Aldrich	3	15
vanillic acid	≥ 97%	Fluka	3	15
Hydroxycoumarins				
aesculetin	≥ 98%	Sigma Aldrich	8	30
scopoletin	≥ 98.5%	Sigma Aldrich	1	30
Flavanols				
(-)- epicatechin	≥ 90%	Sigma Aldrich	7	30
(+)- catechin	≥ 98.5%	Fluka	8	30

n.d. = not detected

Fungal strain, culture condition and sample preparation

In our study, different species of white rot fungi, obtained from the fungal culture collection of the Pathology Lab of Edmund Mach Foundation (TN) were used. In particular, six fungal species belonging to the *Armillaria* genus (e.g. *A. borealis, A. cepistipes, A. gallica; A. mellea; A. ostoye* and *A.tabescens*) and three fungal species belonging to the *Heterobasidion* genus (e.g. *H. abietinum; H. parviporum* and *H. annosum sensu stricto*) were selected. In order to promote the mycelium growth, all fungal species were cultivated on 20 g l^{-1} of PDA plates for about 15 days at room temperature. A total of 27 plates were prepared (three replicates for each fungal species). After fungal growth, the mycelium of each species was cut into small pieces of the same size, using a sterile scalpel blade, then inoculated into 27 sterile glass vials (Sartorius AG, Goettingen, Germany) containing 3 g of sterile silver fir sawdust. Sawdust was prepared by milling sapwood of silver fir with the mechanical mill M20 (IKA–WERKE, Staufen, Germania) to obtain a powder of about 3 mm in diameter. Silver fir sapwood was previously taken from a living tree located in the ‘‘Abeti Soprani" forest in the Molise Region (Italy) using a chainsaw. To promote the fungal growth in silver fir sawdust, 5 ml of sterile ultra-pure water was added in each glass vials (Woods et al. 2005). In addition to the 27 vials with the fungal inoculum, other three vials were prepared as control, containing only sawdust and stored in the freezer (-20 °C). Subsequently, from each vial, 1 g of silver fir sawdust was collected after two (t1), four (t2) and six months (t3), resulting in a total of 81 samples and stored in the freezer. Before the analysis of phenolic compounds, all samples were dried in the oven at 30 °C for one week. After drying, 0.1 g of each sample was transferred into 2-ml Eppendorf tubes and then extracted in 2 ml of water-methanol mixture (75:25, v/v). The solutions were first homogenized for 2 min using an Ultrasonic processor (UP50H; *50 watts*, *30 kHz;* Hielscher Ultrasonics GmbH, *Warthestraße*, Germany) and then were shaken using a Multi Reax (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) for 15 min. The samples were centrifuged at 15,000 rpm and 10 °C for 5 min and then suspended again with the Multi Reax; afterwards, they were left to rest for one hour. Finally,

all samples were centrifuged for 5 min at 15,000 rpm and the supernatant was filtered with 0.45 μm PTFE filter cartridges (Sartorius AG) into an analytical 2 ml vial, and 10 μl injected.

Evaluation of sample preparation

The extraction procedure is a main step for the quantification of wood constituents, such as phenolic compounds. Extraction yield of phenolics is affected by several factors, such as the solvents used with varying polarities, sample-to-solvent ratio, extraction time, temperature, and the characteristics of the sample (Robards et al. 2003). Studies have reported that different solvents, such as methanol, ethanol, acetone, ethyl acetate, and their combinations with different proportions of water were used for the extraction of phenolic from wood and plant materials (Pinelo et al. 2005; Gironi et al. 2011). In this study, the extraction of phenolic compounds from silver fir sawdust containing the fungal inoculum was evaluated at different temperatures (30 and 80 °C) using various mixes of methanol/water (100% H_2O; H_2O/MeOH at 75:25, 50:50, 25:75; 100% MeOH).

LC-HRMS analysis

The analysis of phenolic compounds was carried out according to the method described by Barnaba et al. (2018). In particular, the identification and quantification of these compounds was performed using a Thermo Ultimate™ 3000 HPLC (Thermo Scientific, Sunnyvale,CA, USA) coupled to a hybrid quadrupole-orbitrap mass spectrometer (Q-ExactiveTM; Thermo Scientific, Bremen, Germany), equipped with heated electrospray ionization (HESI-II). Chromatographic separation was carried out by injecting 10 μl of sample on an Accucore™ Polar Premium LC column (150 mm × 3 mm, 2.6 μm particle size; Thermo Fischer Scientific, Waltham, MA, USA), using water-acetonitrile gradient at a flow rate of 0.3 ml min^{-1}. Mass detection was performed in negative ion mode using a full MS-data dependent MS/MS analysis (full MS–dd MS/MS). Full mass spectra were recorded with a resolution of 70,000 full width at half-maximum (FWHM, calculated for *m/z* 200, 1.5 Hz), automatic gain control (AGC) target of $5 \cdot 10^5$ ions and maximum inject time (IT) 150 ms. Data-dependent mass spectra were recorded with a resolution of 17,500 FWHM (*m/z* 200, 12 Hz), AGC target $1 \cdot 10^5$ of ions and IT of 50 ms. The mass spectrometer operated using the following parameters:

spray voltage, 2.80 kV; sheath gas flow rate, 30 arbitrary units; auxiliary gas flow rate, 20 arbitrary units; capillary temperature, 310 °C; capillary gas heater temperature, 280 °C. Data acquisition and processing were carried out with Thermo Scientific™ Dionex™ Chromeleon™ 7.2 Chromatography Data System (CDS) software.

Method validation

The characteristics of the method were studied using the 18 pure standards corresponding to the phenolic compounds quantified in the analysed samples (Table 2). Limits of quantification (LOQ) were established as ten standard deviations of ten replicated blank samples according to EURACHEM (1993). Method trueness was estimated as recovery (%) of one sample spiked at two increasing concentration levels, covering the quantitation range of each phenol (high concentration, 1 mg l^{-1}; low concentration, 0.2 mg l^{-1}) each one analytically replicated three times. Precision was assessed as the relative standard deviation (RSD%) of the same experimental samples used for calculating trueness.

Statistical analysis

Statistical analysis was performed using Statistica 13.1 (Stat- Soft, 2010) and XLSTAT (version 2018.1 Addinsoft, France). Significant differences between the concentrations of phenolic compounds measured during different times of degradation were determined using Tukey's HSD test ($p < 0.05$). Principal component analysis (PCA) was carried out to evaluate the relationhips between phenolic compounds and the activity of fungal species during different times of silver fir wood degradation.

Results and discussion

Evaluation of sample preparation

The use of different temperatures (30 and 80 °C) to dry the sawdust samples did not influence the final content of phenolic compounds in the extracted samples. However, several studies showed that the use of high temperatures in the drying process might promote possible concurrent degradation of phenolic compounds (Chan et al. 2009; Mokrani et al. 2016). For this reason, all samples were dried in the oven at 30 °C. As regards the solvent mixes, the combination of different solvents produced different recoveries of phenolic compounds and the best compromise was obtained using H_2O/MeOH at 75:25, which was able also to optimise the extraction of gallic acid, 4-ethylguaiacol, eugenol and epicatechin.

Method validation

The method characteristics including LOQ, linearity range, precision and trueness determined for each phenolic compound, are shown in Table 2. Trueness, in the samples added with 0.2 mg/l of standard mix solution evaluated in terms of recovery, was between 70 and 120% for acetosyringone, isoacetosyringone, pyrocatechol and vanillic acid, while from 40 to 70% for the remaining ones. In the samples added with 1 mg/l of standard mix solution, the recovery (%) was between 70 and 120% for all the phenols, except for coniferyl alcohol, coniferylaldehyde, ferulic acid, scopoletin and vanillic acid (65%, 51%, 51%, 60% and 58%, respectively). The precision values (expressed as relative standard deviations; RSD %) were always below 10% for the samples added with 1 mg/l of

standard mix solution, while for those added with 0.2 mg l^{-1} the values were always being lower than 10%, except for acetovanillone, pyrocatechol, protocatechuic acid and syringic acid (12%, 14%, 16% and 15%, respectively).

Table 2: Validation parameters of 18 phenolic compounds detected in silver fir sawdust samples for LC-Q-Orbitrap analysis.

Phenolic compounds	(m/z)	RT (min)	LOQ	Linearity range ($\mu g\ mL^{-1}$)	Precision (RSD %)l		Accuracy (%)	
					0.2 mg/l	1 mg/l	0.2 mg/	1 mg/l
Simple phenol								
pyrocatechol	1.090.295	10.60	0.0005	0.0005-8.95	16	2	71	82
Alkyphenyl alcohols								
coniferyl alcohol	1.790.714	17.83	0.0107	0.0107-5.35	8	8	43	51
Hydroxybenzoketones								
acetosyringone	1.950.662	19.50	0.0001	0.0001-5.19	9	3	73	92
acetovanillone	1.650.557	20.00	0.0001	0.0001-5.12	12	2	45	76
isoacetosyringone	1.950.662	20.70	0.0001	0.0011-9.72	9	3	73	92
methyl vanillate	1.810.506	29.90	0.0005	0.0005-9.27	8	2	59	69
Hydroxycinnamaldehydes								
coniferylaldehyde	1.770.556	26.20	0.0001	0.0001-5.07	2	4	43	51
Hydroxybenzaldehydes								
syringaldehyde	1.810.506	24.78	0.0008	0.0008-13.5	2	4	55	90
vanillin	1.510.401	17.10	0.0001	0.0001-5.36	3	6	53	71
Hydroxyphenylacetic acids								
homovanillic acid	1.810.506	12.08	0.0010	0.0010-2.97	8	4	52	79
Hydroxycinnamic acids								
ferulic acid	1.930.506	26.01	0.0001	0.0001-6.21	5	3	47	51
p-coumaric acid	1.630.401	29.61	0.0001	0.0001-5.20	7	4	41	74
Hydroxybenzoic acids								
benzoic acid	1.210.295	30.00	0.001	0.001-5	10	1	49	90
p-carboxyphenol acid	1.370.244	18.10	0.0001	0.0001-5.28	7	3	67	106
protocatechuic acid	1.530.193	10.50	0.0001	0.0001-5.03	16	2	82	105
syringic acid	1.970.455	14.92	0.0001	0.0001-4.26	15	1	57	80
vanillic acid	1.670.350	17.10	0.0001	0.0001-3.04	10	2	77	58
Hydroxycoumarins								
scopoletin	1.910.350	21.50	0.0010	0.0010-9.11	11	3	40	60

Note: RT= retention time; LOQ= limit of quantification.*Linerity range and LOQs are defined without considering sample dilution.

Simple phenolic compounds in silver fir wood

Several phenolic compounds were identified in silver fir wood samples. As reported in Table 3 simple phenols (pyrocatechol), alkylphenyl alcohols (coniferyl alcohol), hydroxybenzoketones (acetovanillone and methyl vanillate), hydroxybenzaldehydes (vanillin), hydroxyphenylacetic acids (homovanillic acid), hydroxycinnamic acids (ferulic acid) and hydroxybenzoic acids (benzoic, p-carboxyphenol and vanillic acids) were found in detectable amounts. While acetosyringone, isoacetosyringone, coniferylaldehyde, syringaldeide, p-coumaric acid, protocatechuic acid, syringic acid and scopoletin were always under the quantification limit.

Specifically, among simple phenols, a low content of pyrocatechol (from 0.29 to 0.44 mg kg^{-1}) was detected. As far as we know, this compound was found for the first time in fresh silver fir wood, while other studies showed the production of pyrocatechol after the action of ligninolytic enzymes of white rot fungi during lignin degradation (Hammel et al. 1985; Miki et al. 1986).

As regards alkylphenyl alcohols, a high concentration of coniferyl alcohol (from 5.9 to 13.9 mg kg^{-1}) was found. Coniferyl alcohol is one of the main components of lignin structure, particularly abundant in coniferous species, such as silver fir (Vanholme et al. 2012). Among hydroxybenzaldehydes, notable high concentrations of vanillin (from 39.4 to 58.7 mg kg^{-1}) were found. These results agree with Zarzyński (2009), concerning the identification and quantification of phenolic compounds in wood of exotic and European tree species, such as silver fir. As regards hydroxybenzoketones, acetovanillone and methyl vanillate, they were all found in detectable amount, with concentrations ranging from 2.5 to 3.9 mg kg^{-1} and from 0.15 to 0.20 mg kg^{-1}, respectively. These compounds were detected for the first time in silver fir wood.

Among hydroxycinnamic acids, a low content of ferulic acid (from 0.05 to 0.50 mg kg^{-1}) was found. This compound was previously identified in wood of other coniferous species, such as Norway spruce, by Metsämuuronen and Siren (2014). Finally, as regards hydroxybenzoic acids, benzoic, p-carboxyphenol and vanillic acids were found, with remarkably concentrations ranging from 14.2 to 22.8 mg kg^{-1}, from 4.4 to 6.3 mg kg^{-1} and from 59.3 to 79.7 mg kg^{-1}, respectively.

In particular, among all the phenolic compounds, vanillic acid was the most abundant. Few studies reported the presence of this compound in the bark and wood extract of silver fir (Benković et al. 2014; Tavčar et al. 2017).

Effect of fungal activity on phenolic components

Changes in the phenolic profiles of silver fir wood, in relation to the degradative activity of different fungal species belonging to *Armillaria* and *Heterobasidion* genera, were then evaluated. Different classes of phenolic compounds were identified in wood samples inoculated with various fungal species and resampled in different times during wood degradation. Table 3 summarises the phenolic compound content, while the trend of these compounds during different times of degradation can be observed in Figure 1a, b, c.

Specifically, among simple phenols, pyrocatechol showed a decreasing trend during degradation process in both genera of fungi. This compound was rapidly metabolized by all fungal species, except for *A. gallica*, where the degradation of this compound started only after the second month (t1). Studies conducted on the white rot fungus *Phanerochaete chrysosporium,* highlighted the production of pyrocatechol, due to the oxidation of β-O-4 linkages of lignin, by lignin peroxidase enzyme (Hammel et al. 1985; Miki et al. 1986). However, other studies indicated the decrease of pyrocatechol after the action of laccase enzyme with the subsequent conversion to phenoxyl radicals through oxidation processes (Thurston 1994; Eggert et al. 1996).

As regards alkylphenyl alcohols, coniferyl alcohol showed a decreasing trend during the degradation process in both genera of fungi, with concentrations significantly lower after six months (t3) from fungal inoculation compared to the other degradation times (Table 3). This alcohol is reported as one of the most abundant monolignol of softwood lignin polymer (Önnerud et al. 2002) and several studies described its degradation by fungi and bacteria with the consequent production of ferulic acid, coniferylaldehyde and vanillic acid (Nishida and Fukuzumi 1978; Falconnier et al. 1994; Buraimoh et al. 2017).

Among hydroxybenzoketones, acetosyringone, acetovanillone, isoacetosyringone and methyl-vanillate were found in detectable amounts. Acetosyringone had an interesting accumulation after two months (t1) from fungal inoculation, specifically in the samples inoculated with *Armillaria* species, such as *A. ostoyae* (from 6.7 to 11.4 mg kg^{-1}), and a decrease in further decay times. Kirk and Farrell (1987) reported the presence of this compound in decayed wood after biological degradation of lignin by fungi. As regards acetovanillone, several studies reported the presence of this compound during the lignin degradation by white rot fungi (Johannes and Majcherczyk 2000; Barneto et al. 2012). In our study, acetovanillone showed a significantly decreasing trend during different times of degradation, particularly in the samples inoculated with *A. mellea* and *A. gallica* (Figure 1a). Regarding isoacetosyringone, as far as we know, it was detected for the first time as a lignin product during fungal degradation. After six months (t3), a low content of isoacetosyringone was produced by all fungal species, except for A. *gallica* and A. *cepistipes*. Regarding methyl-vanillate, Abdelaziz et al. (2016) and Yan et al. (2016) reported the presence of this compound among the pyrolysis products of lignins, but no one indicated its production during the lignin degradation by fungi. However, in all samples, we observed a decreasing trend of methyl-vanillate during different times of degradation, with concentrations significantly higher after two months (t1) from fungal inoculation (Table 3).

As regards hydroxycinnamaldehydes, coniferylaldehyde was produced in both genera of fungi after two months from their inoculation in silver fir sawdust and showing a decreasing trend during degradation process. Several studies reported the production of this compound after the enzymatic degradation of coniferyl alcohol by white rot fungi (Boerjan et al. 2003; Buraimoh et al. 2017). Moreover, Falconnier et al. (1994) observed the formation of coniferylaldehyde after the oxidation of the propenoic side chain of ferulic acid by the action of ligninolytic enzymes in a white rot fungus *Trametes versicolor*.

Among hydroxybenzaldehydes, syringaldehyde and vanillin were quantified. In particular, a low content of syringaldehyde was detected only in the samples inoculated with *A. gallica* (from 0.13 to

0.16 mg kg^{-1}), *A. ostoyae* (from 0.04 to 0.07 mg kg^{-1}) and *A. tabescens* (from 0.02 to 003 mg kg^{-1}) after two months (t1) from their inoculation. Moreover, this compound showed a decreasing trend during degradation process. Kirk and Farrell (1987) reported the presence of syringaldehyde after biological degradation of lignin in decayed wood. As regards vanillin, a complete degradation of this compound was observed during decay process in both genera of fungi. Several studies reported the production of vanillin during lignin degradation by different species of basidiomycete fungi (Ghosh and Nanda 1994; Falconnier et al. 1994; Tsujiyama and Ueno 2008). Moreover, in some fungal species vanillin was described as an intermediate during lignin degradation for the conversion of ferulic acid to vanillic acid (Ghosh and Nanda 1994; Tsujiyama and Ueno 2008).

Among hydroxyphenylacetic acids, homovanillic acid was found in detectable amounts. In particular, in the samples inoculated with *Armillaria* species, homovanillic acid showed a decreasing trend during different times of degradation, except in the samples inoculated with *A. tabescens*. In these samples, homovanillic acid increased as degradation proceeded. As regards the samples inoculated with *Heterobasidion* species, an accumulation of homovanillic acid was observed after two months (t1) from fungal inoculation (Table 3) and a decrease in further decay times. Takada et al. (2004) reported that homovanillic acid was produced after lignin degradation by fungi.

Among hydroxycinnamic acids, ferulic and p-coumaric acid were detected. Several studies reported the presence of these compounds after the action of lignin-degrading enzymes (Ander et al. 1984; Ruttimann-Johnson et al. 1996; Tsujiyama et al. 2008; Abdelaziz et al. 2016). In particular, a significantly decreasing trend of ferulic acid was observed for both genera of fungi, but it was more evident in the samples inoculated with *Armillaria* species, such as *A. ostoyae* (Table 3). In literature, different pathways of ferulic acid degradation induced by different fungal species were reported (Falconnier et al. 1994; Krings et al. 2001; Mathew et al. 2007) with the formation of other phenolic compounds, such as vanillic acid and coniferylaldehyde (Falconnier et al. 1994). Regarding *p*-coumaric acid, a high content of this compound was detected after two month (t1) from fungal inoculation in both genera of fungi. Several studies reported different strategies of *p*-coumaric acid

degradation by various fungal species (Alvarado et al. 2001; Torres and Rosazza 2001; Sachan et al. 2006). For example, in the basidiomycete *P. cinnabarinus*, the oxidative degradation of the *p*-coumaric acid side-chain led to the formation of *p*-hydroxybenzoic acid (Alvarado et al. 2001).

As regards hydroxybenzoic acids, benzoic acid, *p*-carboxyphenol acid, protocatechuic acid, syringic acid and vanillic acid were detected in all samples. In previous studies, these compounds were also identified from the decayed woods, as products of the lignin degradation (Chen et al. 1982a; Chen et al. 1983; Wang et al. 2018). In particular, benzoic acid, *p*-carboxyphenol acid and vanillic acid showed a deceasing trend during different times of degradation, in both genera of fungi. Several studies reported that benzoic and vanillic acids might arise after the oxidative cleavage of the α and β carbons of alkyl side chain by manganese peroxidase enzyme (Hammel and Cullen 2008; Crestini et al. 2010). As regards *p*-carboxyphenol acid, as far as we know, it was found for the first time in silver fir wood during the fungal degradation. In all samples, a decreasing trend of *p*-carboxyphenol acid was observed for both genera of fungi during different times of degradation. Regarding protocatechuic acid and syringic acid, an interesting accumulation of these compounds was observed after six months (t3) from fungal inoculation in both genera of fungi. In particular, the concentrations of syringic acid were higher in the samples inoculated with *A. gallica* (from 10.9 to 11.5 mg kg^{-1}) and *H. abietinum* (from 11.6 to 12.1 mg kg^{-1}) compared to the other fungal species. A few studies reported that syringic acid is an intermediate in the lignin degradation by white rot fungi (Haider and Trojanowski 1975; Eriksson et al. 1984). The degradation of syringic acid was studied in detail in the white-rot basidiomycete *Sporotrichum pulverulentum* (Eriksson et al. 1984) through the reduction of the carboxyl group by ligninolytic enzymes. Nakatsubo and Higuchi 1982 showed that syring acid might derive from the conversion of propiosyringone, produced after the degradation by laccase enzyme of phenylcoumarane (β-5) linkages in lignin structure. Moreover, Handerson and Farmer (1955) reported the production of syringic acid also after the oxidation of syringaldehyde by laccase enzymes. As regards protocatechuic acid, a low content of this compound was produced by different fungal species after six months (t3) from their inoculation in silver fir wood (Table 3). A few studies

showed that protocatechuic acid derive from the demethylation of vanillic acid during the metabolism of white rot fungi (Tsujiyama and Ueno 2008).

Finally, among hydroxycoumarins, scopoletin showed an accumulation trend during different times of degradation, with high content in the samples collected after six month (t3) from fungal inoculation (Table 3). As far as we know, scopoletin is produced after the degradation of ferulic acid via the phenylpropanoid pathway (Schoch et al. 2001).

Due to the diversity of lignin chemical structures and enzymatic strategies of lignin depolymerisation by different fungal species, the concentrations of phenolic compounds released from lignin and their trends were variable (Fisher and Fong 2014). According to previous studies, (Mäkelä et al. 2015; Janusz et al. 2017) our results revealed different strategies of phenolic compound degradation in lignin of silver fir wood by different fungal species belonging to *Armillaria* and *Heterobasidion* genera. In particular, the decreasing trend observed for many of the detected phenolic compounds, such as coniferyl alcohol, ferulic acid, p-coumaric acid, acetovanillone, vanillic acid, vanillin, etc., indicated that different fungal species were able to efficiently metabolize most of the phenolic compounds (Guiraud et al. 1999). The accumulation trend of protocatechuic acid, syringic acid and scopoletin could be related to the previous conversion of their phenolic precursors, such as vanillic acid, ferulic acid and syringaldehyde, after the action of ligninolytic enzymes, such as manganese peroxidase, lignin peroxidase and laccase, secreted in both genera of fungi (Campbell 1931; Campbell 1932; Garraway et al. 1991).

Table 3: Statistical distribution of phenolic compound content detected in silver fir sawdust samples during different times of degradation by various fungal species belonging to *Armillaria* and *Heterobasidion* genera. Data are expressed in mg/kg for all phenolic compounds except for isoacetosyringone detected in *Heterobasidion* genus (µg/kg).

Compounds		*Armillaria spp.*																	
	time	*A.borealis*			*A.cepsitipes*			*A.gallica*			*A.mellea*			*A.ostoyae*			*A.tabescens*		
Simple phenols		**Min**	**Mdn**	**Max**	**Min**	**Mdn**	**Max**	**Min**	**Mdn**	**Max**	**Min**	**Mdn**	**Max**	**Min**	**Mdn**	**Max**	**Min**	**Mdn**	**Max**
pyrocatechol	**t0**	0.288	0.322^{a}	0.436	0.288	0.322^{a}	0.436	0.288	0.322^{a}	0.436	0.288	0.322^{a}	0.436	0.288	0.322^{a}	0.436	0.288	0.322^{a}	0.436
	t1	0.020	0.024^{b}	0.024	0.030	0.042^{b}	0.054	0.282	0.296^{a}	0.310	0.034	0.042^{b}	0.048	0.042	0.072^{b}	0.084	0.028	0.054^{b}	0.080
	t2	0.016	0.018^{b}	0.020	0.012	0.015^{b}	0.018	0.022	0.033^{b}	0.044	0.020	0.022^{b}	0.024	0.016	0.023^{b}	0.030	0.022	0.031^{b}	0.040
	t3	<LOQ	<LOQ^{b}	<LOQ	<LOQ	<LOQ^{b}	<LOQ	<LOQ	<LOQ^{b}	<LOQ	<LOQ	<LOQ^{b}	<LOQ	<LOQ	<LOQ^{b}	<LOQ	<LOQ	<LOQ^{b}	<LOQ
Alkylphenyl alcohols																			
coniferyl alcohol	**t0**	5.94	9.93^{a}	13.9	5.94	9.93^{a}	13.9	5.94	9.93^{a}	13.9	5.94	9.93^{a}	13.9	5.94	9.93^{a}	13.9	5.94	9.93^{a}	13.9
	t1	8.01	848^{a}	9.51	7.35	8.55^{a}	11.4	5.15	7.45^{a}	7.50	12.0	12.7^{a}	14.2	6.35	9.11^{a}	10.8	8.13	9.30^{a}	13.1
	t2	5.86	5.96^{a}	8.22	5.54	8.63^{a}	11.1	4.75	4.79^{ab}	7.17	7.83	7.85^{a}	8.35	7.74	8.05^{a}	8.36	6.74	8.00^{a}	11.5
	t3	0.722	1.03^{b}	1.11	0.372	0.674^{b}	0.944	0.372	0.802^{b}	0.934	0.484	0.828^{b}	0.832	0.726	1.37^{b}	1.46	0.536	0.722^{b}	0.790
Hydroxybenzoketones																			
acetosyringone	**t0**	<LOQ	<LOQ^{b}	<LOQ	<LOQ	<LOQ^{b}	<LOQ	<LOQ	<LOQ^{a}	<LOQ	<LOQ	<LOQ^{a}	<LOQ	<LOQ	<LOQ^{b}	<LOQ	<LOQ	<LOQ^{b}	<LOQ
	t1	1.71	1.96^{a}	1.99	1.48	1.83^{a}	2.69	0.136	0.332^{a}	0.766	0.882	0.980^{a}	1.24	6.71	7.87^{a}	11.4	1.60	2.18^{a}	2.75
	t2	1.11	1.40^{b}	1.41	0.870	1.46^{b}	1.07	<LOQ	<LOQ^{a}	<LOQ	0.348	0.426^{a}	0.766	0.874	0.938^{b}	1.43	0.462	0.484^{b}	0.562
	t3	<LOQ	<LOQ^{b}	<LOQ	<LOQ	<LOQ^{b}	<LOQ	<LOQ	<LOQ^{a}	<LOQ	<LOQ	<LOQ^{a}	<LOQ	<LOQ	<LOQ^{b}	<LOQ	<LOQ	<LOQ^{b}	<LOQ
acetovanillone	**t0**	2.51	3.20^{a}	3.89	2.51	3.20^{a}	3.89	2.51	3.20^{a}	3.89	2.51	3.20^{a}	3.89	2.51	3.20^{a}	3.89	2.51	3.20^{a}	3.89
	t1	2.38	2.95^{ab}	3.27	1.41	2.06^{a}	2.71	2.30	2.49^{ab}	2.68	1.80	2.02^{b}	2.25	1.36	2.56^{a}	2.76	2.37	2.57^{a}	2.76
	t2	2.23	2.43^{ab}	2.77	0.334	0.524^{b}	0.534	1.00	1.17^{c}	1.31	0.302	0.312^{c}	1.16	0.302	0.348^{b}	0.364	0.616	0.732^{b}	1.49
	t3	1.56	1.66^{b}	1.90	0.180	0.326^{b}	0.344	1.31	1.50^{bc}	1.71	<LOQ	<LOQ^{c}	<LOQ	<LOQ	0.212^{b}	0.260	0.526	0.532^{b}	0.680
isoacetosyringone	**t0**	<LOQ	<LOQ^{b}	<LOQ	<LOQ	<LOQ^{b}	<LOQ	<LOQ	<LOQ^{b}	<LOQ	<LOQ	<LOQ^{b}	<LOQ	<LOQ	<LOQ^{b}	<LOQ	<LOQ	<LOQ^{b}	<LOQ
	t1	<LOQ	<LOQ^{b}	<LOQ	<LOQ	<LOQ^{b}	<LOQ	<LOQ	<LOQ^{b}	<LOQ	<LOQ	<LOQ^{b}	<LOQ	<LOQ	<LOQ^{b}	<LOQ	<LOQ	<LOQ^{b}	<LOQ
	t2	0.01	0.02^{b}	0.03	<LOQ	<LOQ^{b}	<LOQ	<LOQ	<LOQ^{b}	<LOQ	0.002	0.002^{b}	0.003	0.002	0.002^{b}	0.003	<LOQ	0.002^{b}	0.003
	t3	0.868	0.892^{a}	0.916	0.001	0.002^{a}	0.003	0.002	0.002^{a}	0.003	0.247	0.269^{a}	0.278	1.05	1.17^{a}	1.29	0.144	0.160^{a}	0.177
methyl vanillate	**t0**	0.152	0.178^{b}	0.204	0.152	0.178^{ab}	0.204	0.152	0.178^{b}	0.204	0.152	0.178^{a}	0.204	0.152	0.178^{a}	0.204	0.152	0.178^{b}	0.204
	t1	0.452	0.704^{a}	0.778	0.280	0.368^{a}	0.896	0.354	0.744^{a}	0.828	0.328	0.344^{b}	0.390	0.180	0.354^{a}	0.382	0.316	0.378^{a}	0.398
	t2	0.090	0.154^{b}	0.250	0.024	0.032^{b}	0.038	0.040	0.044^{b}	0.050	0.016	0.018^{c}	0.024	0.022	0.028^{b}	0.028	0.072	0.090^{bc}	0.136
	t3	0.046	0.070^{b}	0.080	0.014	0.018^{b}	0.018	0.024	0.036^{b}	0.036	0.016	0.018^{c}	0.020	0.022	0.026^{b}	0.030	0.030	0.032^{c}	0.040
Hydroxycinnamaldehydes																			
coniferylaldehyde	**t0**	<LOQ	<LOQ^{a}	<LOQ	<LOQ	<LOQ^{a}	<LOQ	<LOQ	<LOQ^{c}	<LOQ	<LOQ	<LOQ^{a}	<LOQ	<LOQ	<LOQ^{b}	<LOQ	<LOQ	<LOQ^{c}	<LOQ

	t1	0.844	0.850[a]	0.900	0.588	0.956[a]	1.12	0.896	0.906[a]	0.942	0.440	0.976[a]	1.03	1.20	1.33[a]	1.60	0.664	0.670[a]	1.11
	t2	0.568	0.988[a]	0.988	0.546	0.666[a]	0.708	0.684	0.684[a]	0.728	0.518	0.672[a]	0.712	0.424	1.07[a]	1.20	0.570	0.624[a]	0.766
	t3	0.690	0.776[a]	0.880	0.124	0.244[a]	0.380	0.098	0.204[a]	0.266	0.462	0.488[a]	0.638	0.050	0.832[a]	0.840	0.202	0.252[a]	0.468
Hydroxybenzaldehydes																			
syringaldehyde	**t0**	<LOQ	<LOQ[a]	<LOQ	<LOQ	<LOQ[a]	<LOQ	<LOQ	<LOQ[c]	<LOQ	<LOQ	<LOQ[a]	<LOQ	<LOQ	<LOQ[c]	<LOQ	<LOQ	<LOQ[b]	<LOQ
	t1	<LOQ	<LOQ[a]	<LOQ	0.004	0.004[a]	0.005	0.134	0.140[a]	0.156	<LOQ	<LOQ[a]	<LOQ	0.040	0.042[a]	0.066	0.020	0.028[a]	0.030
	t2	<LOQ	<LOQ[a]	<LOQ	<LOQ	<LOQ[a]	<LOQ	0.112	0.114[b]	0.122	<LOQ	<LOQ[a]	<LOQ	0.016	0.024[b]	0.032	<LOQ	<LOQ[b]	<LOQ
	t3	<LOQ	<LOQ[a]	<LOQ	<LOQ	<LOQ[a]	<LOQ	<LOQ	<LOQ[c]	<LOQ	<LOQ	<LOQ[a]	<LOQ	<LOQ	<LOQ[c]	<LOQ	<LOQ	<LOQ[b]	<LOQ
vanillin	**t0/10**	39.4	49.1[a]	58.7	39.4	49.1[a]	58.7	39.4	49.1[a]	58.7	39.4	49.1[a]	58.7	39.4	49.1[a]	58.7	39.4	49.1[a]	58.7
	t1	2.25	2.46[b]	2.46	2.76	2.93[b]	3.40	7.24	7.89b	8.00	11.3	12.1[b]	12.8	2.11	2.73[b]	3.31	2.23	2.25[b]	3.58
	t2	1.88	2.05[b]	2.07	1.76	2.35[b]	2.47	2.86	3.33[b]	5.14	1.31	1.59[b]	2.92	1.95	1.98[b]	2.01	1.25	1.62[b]	1.83
	t3	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ	1.23	1.69[b]	1.87	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ
Hydroxyphenylacetic acids																			
homovanillic acid	**t0**	0.298	0.490[a]	0.682	0.298	0.490[a]	0.682	0.298	0.490[b]	0.682	0.298	0.490[a]	0.682	0.298	0.490[a]	0.682	0.298	0.490[b]	0.682
	t1	0.148	0.160[b]	0.160	0.030	0.030[b]	0.096	8.75	8.96[a]	9.18	0.080	0.114[ab]	0.488	0.126	0.200[b]	0.252	0.526	0.784[b]	1.24
	t2	0.118	0.130[b]	0.140	0.012	0.046[b]	0.066	0.048	0.072[bc]	0.260	0.072	0.110[ab]	0.124	0.114	0.141[b]	0.168	0.118	0.356[b]	2.30
	t3	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ	0.034	0.036[c]	0.046	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ	3.01	4.32[a]	6.07
Hydroxycinammic acids																			
ferulic acid	**t0**	0.050	0.277[b]	0.504	0.050	0.277[ab]	0.504	0.050	0.277[a]	0.504	0.050	0.277[ab]	0.504	0.050	0.277[bc]	0.504	0.050	0.277[a]	0.504
	t1	0.904	1.02[a]	1.10	0.290	0.736[a]	1.13	0.222	0.298[a]	0.348	0.380	0.620[a]	0.778	1.44	1.53[a]	2.09	0.256	0.364[a]	1.47
	t2	0.736	0.88[a]	1.28	0.350	0.426[ab]	0.572	0.240	0.254[a]	0.334	0.354	0.388[ab]	0.452	0.490	0.998[b]	1.13	0.160	0.316[a]	0.734
	t3	0.104	0.116[b]	0.148	0.001	0.001[b]	0.01	0.010	0.030[a]	0.038	0.070	0.122[b]	0.208	0.052	0.054[c]	0.094	0.210	0.340[a]	0.376
p-coumaric acid	**t0**	<LOQ	<LOQ[c]	<LOQ	<LOQ	<LOQ[c]	<LOQ	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[a]	<LOQ	<LOQ	<LOQ[c]	<LOQ	<LOQ	<LOQ[b]	<LOQ
	t1	0.952	0.998[a]	0.998	0.428	0.578[a]	0.654	0.144	0.310[a]	0.400	0.156	0.836[a]	1.51	0.748	1.07[a]	1.16	0.156	0.168[a]	0.274
	t2	0.724	0.728[b]	0.893	0.326	0.364[b]	0.444	0.104	0.174[ab]	0.186	0.324	0.388[a]	0.684	0.288	0.476[b]	0.526	0.084	0.089[b]	0.094
	t3	<LOQ	<LOQ[c]	<LOQ	<LOQ	<LOQ[c]	<LOQ	0.106	0.110[ab]	0.140	0.020	0.024[a]	0.032	<LOQ	<LOQ[c]	<LOQ	0.014	0.018[b]	0.056
Hydroxybenzoic acids																			
benzoic acid	**t0**	14.2	17.8[a]	22.8	14.2	17.8[a]	22.8	14.2	17.8[a]	22.8	14.2	17.8[a]	22.8	14.2	17.8[a]	22.8	14.2	17.8[a]	22.8
	t1	7.36	8.12[b]	8.72	5.72	10.1[b]	10.5	4.15	4.54[b]	4.56	7.60	7.79[b]	7.80	2.54	3.11[b]	13.9	7.57	7.72[b]	8.52
	t2	4.05	4.13[b]	4.45	5.86	6.28[b]	7.10	3.33	3.61[b]	4.76	3.09	3.96[b]	4.26	6.51	6.61[b]	7.57	3.45	4.05[b]	4.21
	t3	2.36	2.96[b]	4.35	2.12	3.06[b]	4.19	2.15	2.46[b]	3.29	2.88	3.45[b]	3.55	2.47	2.58[b]	4.21	2.78	2.78[b]	3.27
p-carboxyphenol acid	**t0**	4.41	5.37[a]	6.33	4.41	5.37[a]	6.33	4.41	5.37[a]	6.33	4.41	5.37[a]	6.33	4.41	5.37[a]	6.33	4.41	5.37[a]	6.33
	t1	2.00	2.06[b]	2.12	0.382	0.448[b]	0.602	0.698	0.912[b]	1.87	0.302	0.324[b]	0.464	1.24	1.26[b]	1.28	0.352	0.410[b]	0.516
	t2	0.350	0.354[c]	0.376	0.350	0.410[b]	0.600	0.542	0.604[b]	0.612	0.224	0.331[b]	0.438	0.218	0.415[b]	0.612	0.186	0.280[b]	0.406
	t3	0.294	0.308[c]	0.384	0.280	0.398[b]	0.466	0.072	0.144[b]	0.148	0.162	0.200[b]	0.212	0.214	0.287[b]	0.360	0.134	0.164[b]	0.224
protocatechuic acid	**t0**	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[a]	<LOQ	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ
	t1	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[a]	<LOQ	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ
	t2	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[a]	<LOQ	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ
	t3	0.002	0.002[a]	0.002	0.01	0.01[a]	0.07	0.01	0.01[a]	0.027	0.001	0.001[a]	0.003	0.002	0.002[a]	0.004	0.001	0.003[a]	0.004
syringic acid	**t0**	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ
	t1	0.126	0.127[b]	0.128	0.152	0.186[b]	0.190	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ	0.316	0.322[b]	0.590
	t2	0.148	0.208[b]	0.216	0.106	0.204[b]	0.252	0.122	0.200[b]	0.308	0.060	0.078[b]	0.106	0.060	0.069[b]	0.078	0.136	0.168[b]	0.200

	t3	7.93	10.4[a]	11.5	6.02	8.52[a]	11.0	10.9	11.2[a]	11.5	4.03	6.04[a]	8.08	6.57	7.11[a]	7.65	5.99	6.93[a]	7.05
vanillic acid	**t0/10**	59.3	69.5[a]	79.7	59.3	69.5[a]	79.7	59.3	69.5[a]	79.7	59.3	69.5[a]	79.7	59.3	69.5[a]	79.7	59.3	69.5[a]	79.7
	t1	8.55	8.93[b]	9.56	20.5	23.4[b]	26.2	26.1	35.7[b]	45.4	7.31	7.73[b]	8.50	7.28	8.74[b]	10.2	8.39	10.5[b]	10.7
	t2	8.22	8.44[b]	8.61	11.5	12.5[bc]	12.7	10.6	13.3[c]	13.8	4.62	4.77[b]	4.77	4.50	4.76[b]	5.88	6.13	6.75[b]	7.18
	t3	1.74	2.10[b]	2.30	1.45	1.92c	3.61	5.07	5.67[c]	6.27	1.04	1.58[b]	1.69	2.07	3.09[b]	3.27	1.36	1.64[b]	2.07
Hydroxycoumarins																			
scopoletin	**t0**	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[c]	<LOQ	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[c]	<LOQ	<LOQ	<LOQ[b]	<LOQ
	t1	0.001	0.004[b]	0.004	0.002	0.003[c]	0.004	0.016	0.018[b]	0.020	0.002	0.002[b]	0.006	0.004	0.004[c]	0.008	0.004	0.006[b]	0.008
	t2	0.006	0.006[b]	0.006	0.056	0.134[b]	0.152	0.022	0.042[b]	0.062	0.002	0.048[b]	0.094	0.200	0.202[b]	0.210	0.012	0.014[b]	0.016
	t3	0.102	0.146[a]	0.152	0.212	0.273[a]	0.334	1.05	1.37[a]	2.40	0.144	0.154[a]	0.212	0.368	0.480[a]	0.592	0.118	0.120[a]	0.172

		Heterobasidion spp								
		H.abietinum			***H.parviporum***			***H.annosum***		
Compounds	**time**	**Min**	**Mdn**	**Max**	**Min**	**Mdn**	**Max**	**Min**	**Mdn**	**Max**
Simple phenol										
pyrocatechol	**t0**	0.288	0.322[a]	0.436	0.288[a]	0.322	0.436	0.288	0.322[a]	0.436
	t1	0.060	0.070[b]	0.088	0.058	0.080[b]	0.086	0.024	0.026[b]	0.032
	t2	0.012	0.015[b]	0.018	0.026	0.035[b]	0.044	0.006	0.011[b]	0.016
	t3	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ
Alkylphenyl alcohols										
coniferyl alcohol	**t0**	5.94	9.93[a]	13.9	5.94	9.93[a]	13.9	5.94	9.93[a]	13.9
	t1	7.53	7.61[a]	8.89	4.31	8.88[a]	9.17	6.43	7.44[ab]	11.7
	t2	6.43	6.95[a]	7.27	5.74	7.08[ab]	7.51	4.29	9.54[ab]	11.1
	t3	0.406	0.724[b]	0.814	0.456	0.924[b]	0.968	0.410	1.12[b]	1.14
Hydroxybenzoketones										
acetosyringone	**t0**	<LOQ	<LOQ[a]	<LOQ	<LOQ	<LOQ[a]	<LOQ	<LOQ	<LOQ[a]	<LOQ
	t1	0.030	0.096[a]	0.162	0.134	0.170[a]	0.182	0.090	0.120[a]	0.214
	t2	<LOQ	<LOQ[a]	<LOQ	<LOQ	<LOQ[a]	<LOQ	<LOQ	<LOQ[a]	<LOQ
	t3	<LOQ	<LOQ[a]	<LOQ	<LOQ	<LOQ[a]	<LOQ	<LOQ	<LOQ[a]	<LOQ
acetovanillone	**t0**	2.51	3.20[a]	3.89	2.51	3.20[a]	3.89	2.51	3.20[a]	3.89
	t1	2.13	2.29[ab]	2.57	3.43	4.15[a]	4.37	2.93	3.07[a]	4.18
	t2	1.64	2.00[ab]	2.86	1.00	1.40[b]	1.67	0.180	0.326[b]	0.368
	t3	0.964	1.23[b]	1.74	0.994	1.66[b]	2.10	0.136	0.170[b]	0.334
isoacetosyringone	**t0**	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ
	t1	<LOQ	<LOQ [b]	<LOQ	<LOQ	<LOQ [b]	<LOQ	n.d.	<LOQ[b]	<LOQ
	t2	<LOQ	<LOQ [b]	<LOQ	<LOQ	<LOQ [b]	<LOQ	n.d.	<LOQ[b]	<LOQ
	t3	0.002	0.002[a]	0.002	0.001	0.002[a]	0.003	0.001	0.001[a]	0.002
methyl vanillate	**t0**	0.152	0.178[b]	0.204	0.152	0.178[b]	0.204	0.152	0.178[b]	0.204
	t1	0.474	1.00[a]	1.16	0.636	1.01[a]	1.30	0.226	0.298[a]	0.4
	t2	0.030	0.030[b]	0.044	0.024	0.0028[b]	0.036	0.016	0.022[c]	0.03
	t3	0.034	0.034[b]	0.038	0.026	0.026[b]	0.032	0.012	0.012[c]	0.02
Hydroxycinnamaldehydes										

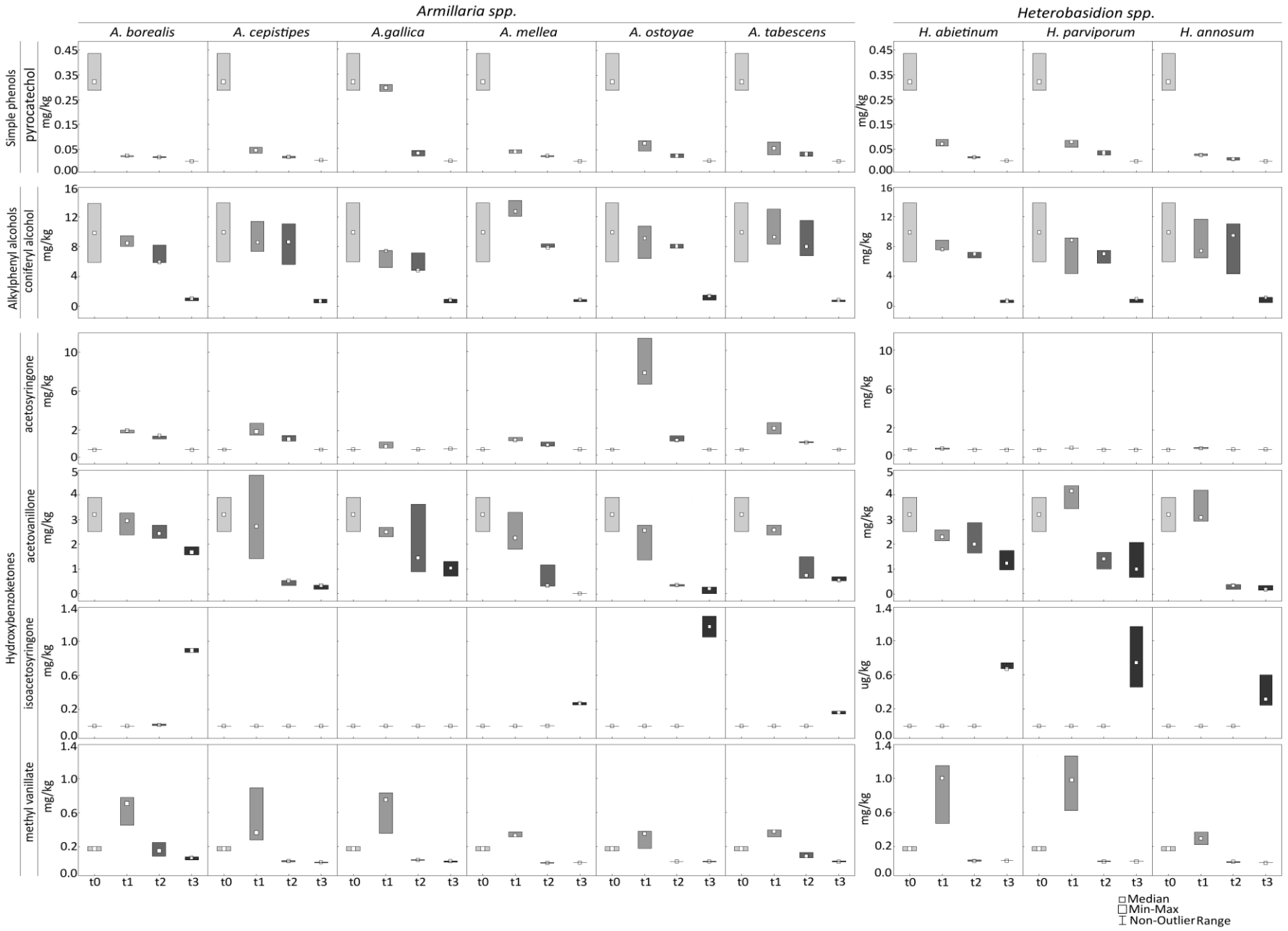
coniferylaldehyde	**t0**	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ
	t1	0.708	0.730[a]	1.28	0.846	0.858[a]	0.914	0.576	0.598[a]	0.752
	t2	0.756	0.770[a]	0.854	0.570	0.600[a]	0.916	0.354	0.508[a]	0.508
	t3	0.096	0.166[a]	0.184	0.116	0.158[a]	0.246	0.050	0.064[a]	0.166
Hydroxybenzaldehydes										
syringaldehyde	**t0**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	t1	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	t2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	t3	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
vanillin	**t0/10**	39.4	49.1[a]	58.7	39.4	49.1[a]	58.7	39.4	49.1[a]	58.7
	t1	34.3	38.0[b]	42.8	5.01	5.50[b]	8.14	2.07	2.25[b]	3.20
	t2	8.19	8.70[b]	9.42	2.85	3.85[b]	4.90	1.67	1.83[b]	1.88
	t3	5.96	6.13[b]	6.48	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ
Hydroxyphenylacetic acids										
homovanillic acid	**t0**	0.298	0.490[b]	0.682	0.298	0.490[b]	0.682	0.298	0.490[b]	0.682
	t1	5.19	6.29[a]	7.39	8.46	9.58[a]	10.7	7.85	8.90[a]	9.95
	t2	0.028	0.034[b]	0.062	0.016	0.032[b]	0.034	0.072	0.084[b]	0.232
	t3	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ
Hydroxycinnamic acid										
ferulic acid	**t0**	0.050	0.277[a]	0.504	0.050	0.277[a]	0.504	0.050	0.277[a]	0.504
	t1	0.160	0.172[a]	0.178	0.138	0.164[a]	0.238	0.022	0.100[a]	0.198
	t2	0.014	0.136[a]	0.164	0.048	0.124[a]	0.180	0.048	0.082[a]	0.130
	t3	<LOQ	<LOQ[a]	<LOQ	0.020	0.028[a]	0.060	0.034	0.054[a]	0.078
p-coumaric acid	**t0**	<LOQ	<LOQ[a]	<LOQ	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[c]	<LOQ
	t1	0.272	0.273[b]	0.274	0.100	0.350[a]	0.502	0.516[a]	0.528	0.650
	t2	0.138	0.196[c]	0.202	0.084	0.140[ab]	0.238	0.154[b]	0.164	0.260
	t3	0.050	0.056[d]	0.088	0.050	0.114[ab]	0.210	0.016[c]	0.048	0.060
Hydroxybenzoic acids										
benzoic acid	**t0**	14.2	17.8[a]	22.8	14.2	17.8[a]	22.8	14.2	17.8[a]	22.8
	t1	4.96	5.03[b]	5.93	3.29	4.80[b]	5.36	5.85	6.72[b]	7.57
	t2	2.48	2.50[b]	2.51	3.17	4.38[b]	4.69	2.09	3.31[b]	3.59
	t3	3.09	3.44[b]	3.79	1.37	2.02[b]	2.55	1.66	3.17[b]	3.63
p-carboxyphenol acid	**t0**	4.41	5.37[a]	6.33	4.41	5.37[a]	6.33	4.41	5.37[a]	6.33
	t1	0.648	0.710[b]	0.956	0.606	0.772[b]	0.788	0.138	0.555[b]	0.972
	t2	0.574	0.636[b]	0.698	0.552	0.571[b]	0.590	0.188	0.206[b]	0.382
	t3	0.144	0.206[b]	0.244	0.110	0.182[b]	0.306	0.150	0.226[b]	0.234
protocatechuic acid	**t0**	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ
	t1	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ
	t2	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ
	t3	0.026	0.106[a]	0.108	0.014	0.016[a]	0.032	0.008	0.008[a]	0.062
syringic acid	**t0**	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ	<LOQ
	t1	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ	0.114	0.134[b]	0.154

	t2	0.088	0.106[b]	0.124	0.086	0.122[b]	0.136	0.300	0.400[b]	0.500
	t3	11.6	11.8[a]	12.1	6.23	6.48[a]	6.73	6.65	7.76[a]	8.88
vanillic acid	**t0**	59.3	69.5[a]	79.7	59.3	69.5[a]	79.7	59.3	69.5[a]	79.7
	t1	34.3	38.0[b]	42.8	42.9	43.2[b]	43.7	10.6	14.8[b]	15.7
	t2	8.19	8.70[c]	9.42	6.95	7.08[c]	7.49	4.42	4.45[b]	5.11
	t3	5.96	6.22[c]	6.48	3.50	3.64[c]	3.78	1.60	2.70[b]	3.23
Hydroxycoumarins										
scopoletin	**t0**	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[b]	<LOQ	<LOQ	<LOQ[a]	<LOQ
	t1	<LOQ	0.008[b]	0.110	0.016	0.028[b]	0.062	0.010	0.011[a]	0.012
	t2	0.012	0.012[b]	0.278	0.018	0.064[b]	0.068	0.024	0.0049[a]	0.074
	t3	0.700	0.872[a]	0.872	0.712	1.05[a]	1.31	0.158	0.168[a]	0.662

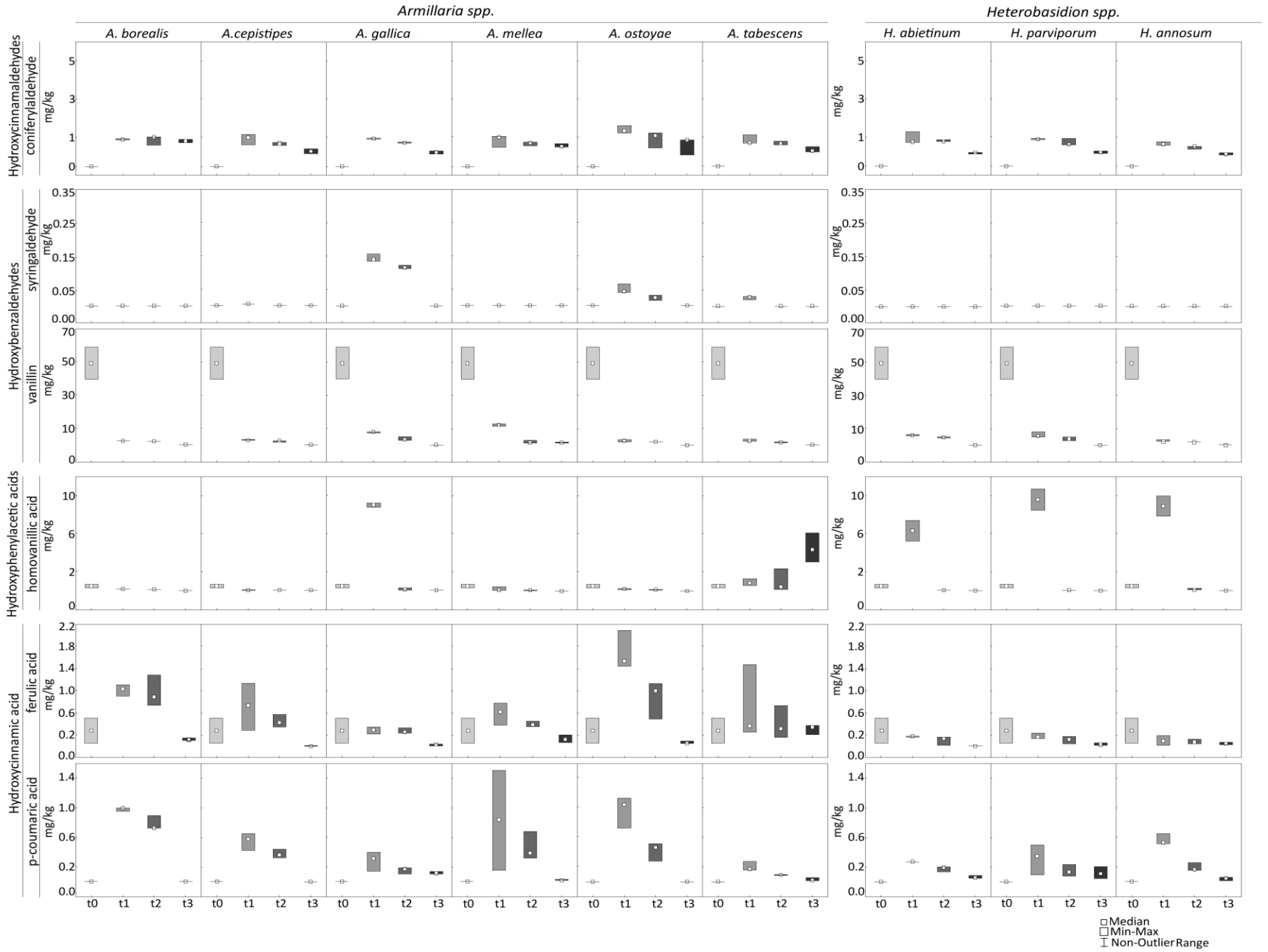
Note: Min = minimum content; Mdn = median content; Max = maximum content; LOQ= limit of quantification.

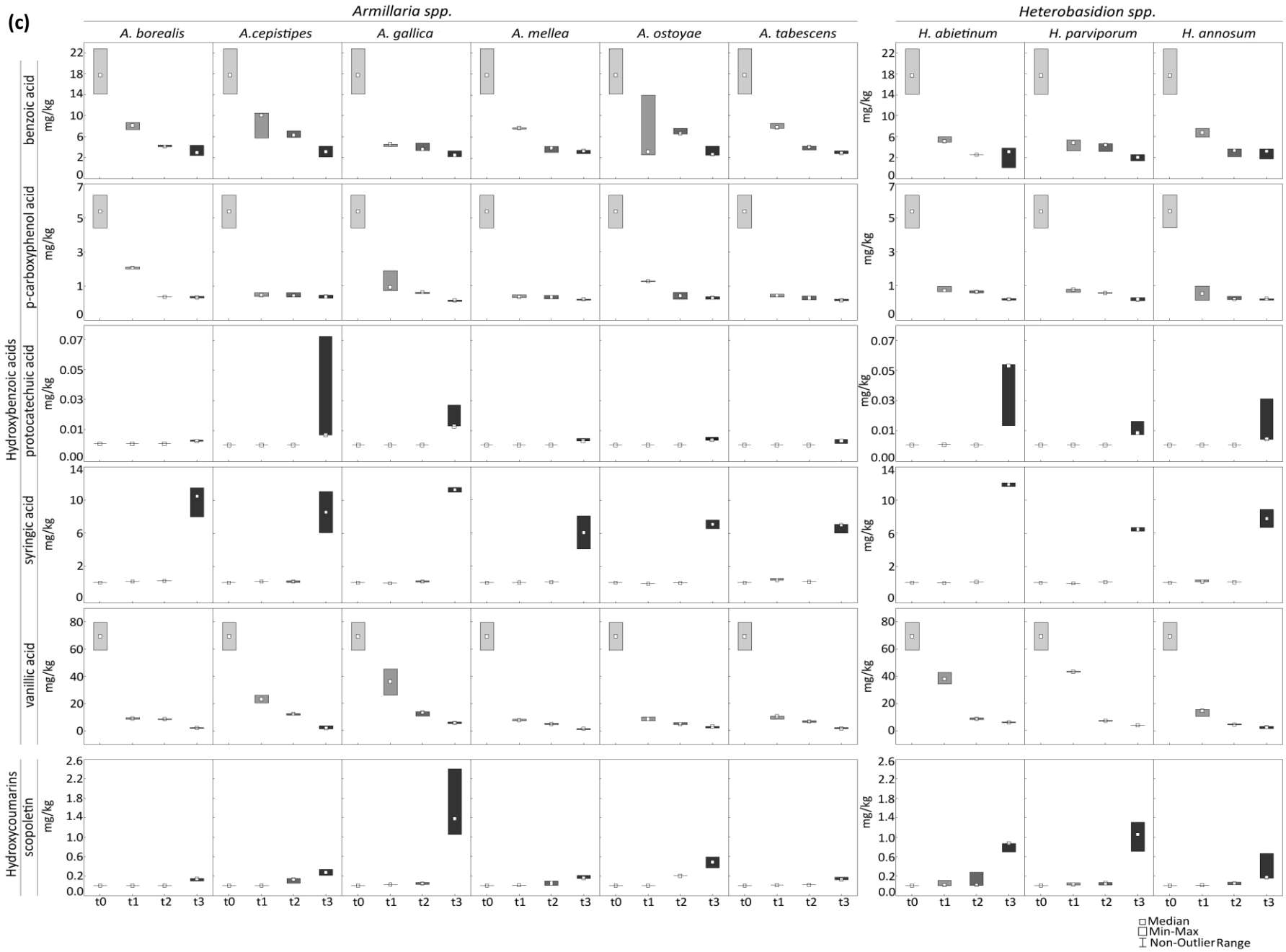
Figure 1 (a,b,c): Box plots with phenolic compounds content (mg/kg and ug/kg) produced by different fungal species belonging to *Armillaria* and *Heterobasidion* genera during times of silver fir sawdust degradation.

(a)


Armillaria spp.
Heterobasidion spp.
A. borealis
A. cepistipes
A.gallica
A. mellea
A. ostoyae
A. tabescens
H. abietinum
H. parviporum
H. annosum
Simple phenols
pyrocatechol
Alkylphenyl alcohols
coniferyl alcohol
Hydroxybenzoketones
acetosyringone
acetovanillone
isoacetosyringone
methyl vanillate
mg/kg
ug/kg
t0
t1
t2
t3
Median
Min-Max
Non-Outlier Range

(b)


Armillaria spp.
Heterobasidion spp.
A. borealis
A.cepistipes
A. gallica
A. mellea
A. ostoyae
A. tabescens
H. abietinum
H. parviporum
H. annosum
Hydroxycinnamaldehydes
coniferylaldehyde
Hydroxybenzaldehydes
syringaldehyde
vanillin
Hydroxyphenylacetic acids
homovanillic acid
Hydroxycinnamic acid
ferulic acid
p-coumaric acid
mg/kg
t0
t1
t2
t3
Median
Min-Max
Non-Outlier Range

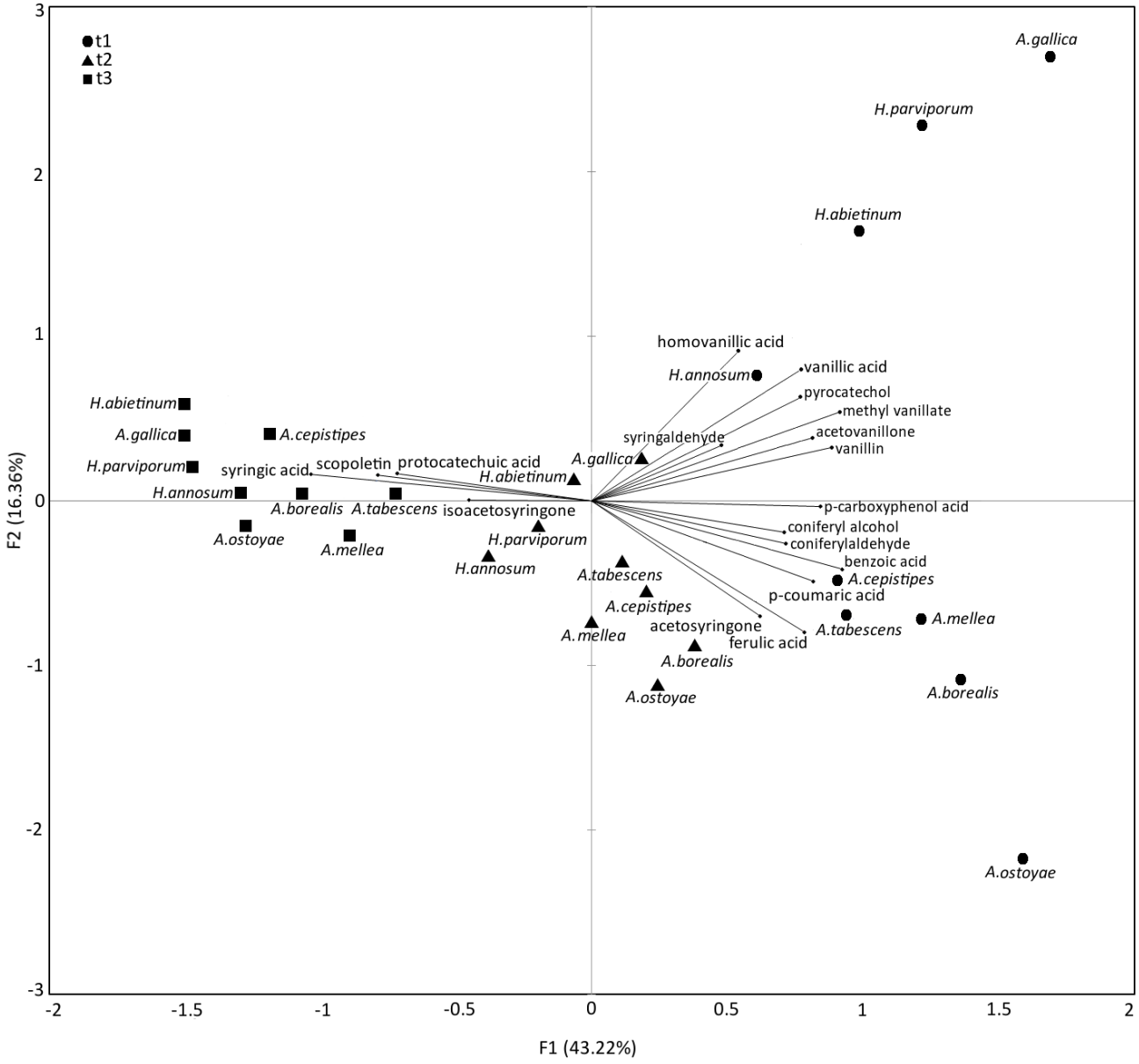

(c)
Armillaria spp.
Heterobasidion spp.
A. borealis
A.cepistipes
A. gallica
A. mellea
A. ostoyae
A. tabescens
H. abietinum
H. parviporum
H. annosum
Hydroxybenzoic acids
benzoic acid
p-carboxyphenol acid
protocatechuic acid
syringic acid
vanillic acid
Hydroxycoumarins
scopoletin
mg/kg
t0
t1
t2
t3
Median
Min-Max
Non-Outlier Range

Phenolic profiles and degradative fungal activity

PCA was applied separately to the phenolic content of silver fir sawdust samples, in order to evaluate the correlations between phenolic profiles and the activity of fungal species during different times of wood degradation. PCA revealed a good differentiation between phenolic compounds and the activity of nine fungal species during silver fir wood degradation (Figure 2), and the variance explained by factor 1 and 2 accounted for 60%. PCA results suggested that most of the phenolic compounds, such as pyrocatechol, coniferyl alcohol, acetovanillone, acetosyringone, methyl vanillate, syringaldehyde, vanillin, homovanillic acid, ferulic acid, p-coumaric acid, benzoic acid, p-carboxyphenol acid, and vanillic acid were rapidly degraded by different fungal species at time (t1) of degradation with diversified concentrations. These results could be explained by the fact that the different fungal species selected in this study are known to attack and metabolize the wood components, such as lignin, during the early times of decay (Ferguson et al. 2003; Zabel and Morrell 1992).

In the following decay times (e.g. t3), the phenolic content did not vary after silver fir wood degradation by different fungal species. In particular, at time (t3) of degradation, various fungal species produced isoacetosyringone, syringic acid, protocatechuic acid and scopoletin.

Figure 2: Principal Component Analysis (PCA) with the distribution of phenolic compounds produced by different fungal species during silver fir sawdust degradation.



Conclusions

In conclusion, the LC-Q-Orbitrap technique proved to be useful for broad identification and sensitive quantification of phenolic compounds present in silver fir wood before and after the inoculation of different fungal species. This study allowed us to elucidate different strategies of lignin degradation in silver fir wood by various fungal species belonging to *Armillaria* and *Heterobasidion* genera. The accumulation and consumption of phenolic compounds during different times of wood decomposition were strictly related to the metabolism of different fungal species and to the activity of ligninolytic enzymes involved in the degradation mechanisms (Hiscox and Boddy 2018).

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CONCLUSIONI
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Conclusioni e prospettive future

Il presente elaborato di tesi, aggiunge informazioni al complesso puzzle dei fattori antropici (es. gestione forestale) e biologici (es. funghi) che influenzano l'abbondanza ed i processi di decomposizione del legno morto di due specie forestali di ampia diffusione in Europa e in Italia: il faggio (*Fagus sylvatica* L.) e l'abete bianco (*Abies alba* Mill.). La crescente attenzione alle funzioni ecologiche del legno morto ed alla sua presenza e distribuzione all'interno degli ecosistemi forestali in relazione alla gestione forestale ed ai tassi di decomposizione, rendono lo studio di tale componente un obiettivo primario, sia per la scelta di opzioni forestali sostenibili volte a mitigare i cambiamenti climatici in corso, sia per comprendere le dinamiche legate al ciclo dei nutrienti e del carbonio. In particolare, nel primo lavoro in cui è stato analizzato l'effetto di diverse opzioni di gestione forestale (es. innovative e tradizionali) sull'abbondanza e distribuzione del legno morto e su altri indicatori di biodiversità (es. microhabitats e vegetazione del sottobosco) è stato possibile osservare un aumento significativo del volume di legno morto (es. ceppaie, snag, alberi morti in piedi e detriti legnosi grossolani o CWD) in seguito all'applicazione dei trattamenti innovativi (CTT). I risultati della ricerca hanno evidenziato pertanto, che la scelta di opzioni selvicolturali innovative ha rappresentato una valida alternativa gestionale per le faggete montane del Mediterraneo in termini di conservazione della biodiversità. Inoltre, in una prospettiva di lungo termine l'aumento delle quantità di legno morto in seguito alle pratiche selvicolturali e degli altri indicatori analizzati (es. microhabitats e vegetazione del sottobosco), potrebbe risultare un pre-requisito utile per migliorare la complessità strutturale e la stabilità di questi ecosistemi forestali, mirando alla scelta di opzioni selvicolturali sostenibili.

Nel secondo lavoro in cui è stato analizzato il profilo degli zuccheri semplici sia nel legno vivo di abete bianco (*Abies alba* Mill.) che nei campioni di legno morto (CWD) raccolti nelle cinque fasi di decadimento di Hunter sono state evidenziate differenze significative negli andamenti e nella variabilità del contenuto degli zuccheri durante l'avanzamento del decadimento del legno morto in campo. Le differenze riscontrate potrebbero essere legate alle diverse condizioni ambientali (temperatura e umidità) che caratterizzano i due siti forestali e che implicano una diversità nella

composizione specifica e nel ruolo degli organismi decompositori come i funghi che attaccano il legno. Tuttavia, ulteriori studi sull'impatto specifico degli organismi decompositori, come batteri, insetti saproxilici e comunità fungine, sarebbero utili per comprendere più in dettaglio la tendenza specifica all'accumulo ed al consumo di tali composti durante il processo di decomposizione del legno. Infine, la tecnica analitica corrispondente ad un cromatografo a scambio ionico, dotato di un rilevatore amperometrico pulsato e da un rilevatore ad aerosol (IC-PAD-CAD) si è dimostrata utile e valida per l'identificazione e la quantificazione degli zuccheri nei campioni analizzati.

Infine, nel terzo lavoro in cui è stato analizzato l'andamento dei composti fenolici utilizzando un cromatografo liquido ad alte prestazioni, accoppiato ad uno spettrometro di massa ibrido quadripolare orbitrap (LC-Q-Orbitrap) è stato possibile osservare diverse strategie di degradazione di tali composti durante i diversi periodi di decomposizione del legno di abete bianco (2-4 e 6 mesi) da parte di nove specie fungine appartenenti ai generi *Armillaria* spp. ed *Heterobasidion* spp. Tale studio ha permesso di ampliare le conoscenze sull'attività enzimatica e sull'influenza di tali organismi durante il processo di degradazione del legno. Inoltre, la tecnica analitica LC-Q-Orbitrap si è dimostrata utile per un'ampia identificazione ed una sensibile quantificazione dei composti fenolici presenti nel legno di abete bianco prima e dopo l'inoculazione dei funghi.

In definitiva, il lavoro svolto durante la tesi ha permesso di ampliare le conoscenze sui fattori (es. gestione forestale, condizioni ambientali e funghi) che influenzano l'abbondanza ed i processi di decomposizione del legno morto sia a livello qualitativo e sia quantitativo, utilizzando diversi approcci sperimentali e avvalendosi di tecniche analitiche innovative cromatografiche. Tuttavia, al fine di favorire una conoscenza esaustiva sulla complessità delle relazioni esistenti tra il legno morto ed i fattori che ne influenzano l'abbondanza ed i processi di decomposizione, sarebbe opportuno effettuare ulteriori ricerche sull'argomento sia a scala locale e sia globale, integrandole e correlandole con quelle già esistenti. A tal proposito, nel corso dei tre anni di dottorato sono state condotte altre due ricerche. In particolare nella prima ricerca, allo scopo di analizzare gli effetti di variabili geografiche (latitudine, esposizione ed altitudine) sulle dinamiche di decomposizione del legno morto

di *Abies alba Mill*. sono state selezionate tre aree di studio ricadenti in zone montane, alpine ed appenniniche lungo un transetto latitudinale in Italia tra cui: la foresta mista di abete bianco e rosso situata nel comune di Telve di Sopra in Trentino, il bosco di "Abeti Soprani" ricadente nel contesto appenninico molisano e la foresta mista di abete bianco e faggio all'interno del Parco Nazionale dell'Aspromonte in Calabria. All'interno di ciascun' area sono stati individuati 5 plots dislocati lungo un transetto altitudinale compreso tra i 1000 ed i 1800 m di quota, nei quali sono stati posizionati 12 blocchi di legno di abete bianco di dimensioni standard 2x5x5 (in totale 60 campioni per sito) a contatto con il suolo minerale. Il protocollo di campionamento definito sulla base dello studio bibliografico ha previsto il prelievo di tre repliche dei blocchi di legno dopo 8 (T1), 16 (T2), 52 (T3) e 104 (T4) settimane. Pertanto, a partire dal maggio del 2016 corrispondente al periodo di rilascio dei legnetti in campo, si è proceduto al prelievo dei campioni nei periodi stabiliti, concludendo la raccolta in campo a maggio del 2018. Le attività proseguiranno con l'analisi dei blocchi di legno di abete bianco, effettuando in particolare analisi chimico-fisiche (es. analisi della lignina e della cellulosa) e ad analisi cromatografiche per l'identificazione e quantificazione dei metaboliti prodotti dalle comunità fungine durante il processo di decomposizione del legno.

Infine, la seconda ricerca ha riguardato lo studio delle dinamiche di decomposizione di *Fagus sylvatica* L. in relazione all'effetto di deposizioni di azoto e fosforo irrorate artificialmente per simulare condizioni ecologico-ambientali ipotizzabili nello scenario del cambiamento climatico in atto, all' interno di in un sito sperimentale localizzato nel comune di Cembra (Trentino Alto Adige). Nel corso del primo anno di dottorato, in tale sito, all'interno di 12 plots di 15 m di raggio sono stati posizionati 9 blocchi di legno di faggio di dimensioni standard 2x5x5 (in totale 108 campioni) a contatto con il suolo minerale e nel luglio 2018, si è proceduto al prelievo delle prime tre repliche di blocchi di legno per un totale di 36 campioni. Le attività proseguiranno con il prelievo dei restanti blocchi di legno di faggio in seguito alle fertilizzazioni di azoto e fosforo secondo il protocollo di campionamento definito dal progetto.

ELENCO PUBBLICAZIONI

Pubblicazioni

***Lombardi F., Di Lella S., Altieri V., Di Benedetto S., Giancola C., Lasserre B., Kutnar L., Tognetti R., Marchetti M.** *Early responses of biodiversity indicators to innovative and traditional silvicultural treatments in mountain beech forests*. Articolo pubblicato sulla rivista "i-forest".

***Stefania Di Lella, Nicola La Porta, Roberto Tognetti, Fabio Lombardi, Tiziana Nardin and Roberto Larcher.** *Characterization of Silver fir wood decay classes using sugar metabolites detected by ion chromatography (IC).* Articolo pubblicato sulla rivista "Journal of Wood Chemistry and Technology".

***Stefania Di Lella, Nicola La Porta, Roberto Tognetti , Fabio Lombardi, Tiziana Nardin and Roberto Larcher.** *Fungal impact in lignin degradation and simple phenols formation in wood of silver fire.* Articolo da sottomettere alla rivista "Holzforschung journal".

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